
UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

# DEVELOPMENT AND OPTIMISATION OF A GROUP-SPECIFIC REAL-TIME RT-PCR ASSAY FOR THE BROAD DETECTION OF THE SIMBU SEROGROUP ORTHOBUNYAVIRUSES

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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*“Umuntu Ngumuntu Ngabantu”*

## Acknowledgements

As long as we are people before we become professionals, it makes absolute sense to start expressing my eternal gratitude to my parents, sister and grandparents, an example since ever, basis of my education, for having given me all the basic values and necessary experiences for me to be the person I am today.

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## Abstract

The Simbu serogroup within the genus *Orthobunyavirus* belongs to the family *Peribunyaviridae* and comprises 32 recognised three-segmented negative-sense single-stranded RNA viruses, divided into two phylogenetic clades. Some members of this group of arthropod-borne viruses, cosmopolitan distributed, cause neurologic disease in humans as well as reproductive and neurologic disease in domestic animals, however, definitive diagnosis always requires laboratorial confirmation. Few real-time RT-PCR assays have been developed for the molecular diagnosis of Simbu serogroup orthobunyaviruses. There are two published methods with broad detection capacity, utilising either a SYBR® Green based chemistry able to recognise viruses from both clades, which is not absolutely specific, or a TaqMan® based chemistry that recognises only clade B viruses. A novel group-specific TaqMan®-based real-time RT-PCR assay was developed, optimised and laboratory validated for the broad detection of the Simbu serogroup orthobunyaviruses. The published genomic data of the Simbu serogroup members were evaluated, and a conserved region, situated in the segment encoding the nucleocapsid protein, was selected to design a universal primer set and a pair of differently labelled hydrolysis probes, which allowed for the distinction between the two phylogenetic clades of the Simbu serogroup. Seven prototype Simbu serogroup isolates were used for the development of the assay, namely *Akabane orthobunyavirus*, *Simbu orthobunyavirus*, *Shuni orthobunyavirus*, *Sathuperi orthobunyavirus*, *Shamonda orthobunyavirus*, Ingwavuma virus and Sabo virus. The primer and probe concentrations in the reaction were optimised. Amplification efficiency was determined for each one of the viruses: AKAV (99%), SIMV (96%), SHUV (96%), SATV (97%), SHAV (84%), INGV (93%) and SABOV (110%). A panel constituted of genetically related, causative agents of abortion in ruminants and arthropod-borne viruses was selected for *in vitro* specificity analysis, and *in silico* analysis was also performed. The assay was shown to be specific, as no cross-reactions were observed either *in vitro* or *in silico*, and sensitive, with a 95% limit of detection ranging from $10^{0,39}$ to $10^{-3,61}$ $TCID_{50}$/reaction, for the detection of Simbu serogroup viruses. The repeatability of the assay was evaluated for both probes detection, using the intra- and inter-run standard deviations and coefficient of variation. This work resulted in a manuscript in submission process to a peer-reviewed journal. In addition, a comprehensive review of the viruses of the Simbu serogroup was carried out, including sites of viral isolation and seroconversion, and a map was generated using a geographic information system tool.



**Keywords:** *Peribunyaviridae*, Molecular diagnosis, TaqMan® assay, Wide spectrum, Phylogenetic clades

## Resumo

O serogrupo Simbu pertence ao género *Orthobunyavirus,* família *Peribunyaviridae,* e é constituído por 32 vírus de RNA tri-segmentado de cadeia simples e polaridade negativa, divididos em duas clades filogenéticas. Alguns membros deste grupo de vírus transmitidos por artrópodes, com distribuição cosmopolita, causam doença neurológica em humanos bem como doença reprodutiva e neurológica em animais domésticos, no entanto, o diagnóstico definitivo requer sempre confirmação laboratorial. Poucos ensaios de RT-PCR em tempo real têm sido desenvolvidos para o diagnóstico molecular destes vírus, ainda assim, existem dois que se destacam pela capacidade de detecção em largo espectro. Um deles, com sistema de detecção de fluorescência baseado na utilização de SYBR® Green, é capaz de detectar vírus das duas clades, mas não é absolutamente específico, e o outro, baseando-se na utilização de sondas TaqMan®, só detecta vírus de uma das clades. Um ensaio de RT-PCR em tempo real grupo-específico, inédito, com sondas TaqMan®, foi desenvolvido, optimizado e caracterizado em termos laboratoriais, para a detecção em largo espectro dos orthobunyavirus do serogrupo Simbu. Os dados publicados referentes ao genoma dos membros do serogrupo foram analisados, e uma região conservada, situada no segmento codificante da proteína da nucleocápdise, foi eleita para o desenho de um par de primers específicos universal, bem como de duas sondas de hidrólise específicas, distintamente marcadas, e que, portanto, permitem a diferenciação entre as clades filogenéticas. Sete isolados de referência foram utilizados no desenvolvimento do ensaio, nomeadamente *Akabane orthobunyavirus*, *Simbu orthobunyavirus*, *Shuni orthobunyavirus*, *Sathuperi orthobunyavirus*, *Shamonda orthobunyavirus*, Ingwavuma virus e Sabo virus e, consequentemente, as concentrações dos primers e sondas na reação foram optimizadas. A eficiência de amplificação foi determinada para cada um dos vírus: AKAV (99%), SIMV (96%), SHUV (96%), SATV (97%), SHAV (84%), INGV (93%) e SABOV (110%). Um painel constituído por vírus geneticamente relacionados, agentes causais de aborto em ruminantes e transmitidos por artrópodes foi seleccionado no sentido de avaliar a especificidade do ensaio *in vitro*, tendo sido também efectuada uma análise *in silico*. O ensaio é específico, visto que não foram observadas reacções cruzadas quer *in vitro* quer *in silico,* e sensível, com um limite de detecção de 95% entre $10^{0,39}$ a $10^{-3,61}$ $TCID_{50}$/reacção, para a detecção de vírus do serogrupo Simbu. A repetibilidade foi avaliada para a detecção com ambas as sondas, pelo cálculo do desvio padrão intra- e inter-corridas bem como do coeficiente de variação. Este trabalho originou um manuscrito em processo de submissão para uma revista científica. Além disso, foi levada a cabo uma revisão bibliográfica do serogrupo Simbu, incluindo locais de isolamento viral e seroconversão, e um mapa inédito desta distribuição foi gerado.



**Palavras-chave:** *Peribunyaviridae*, Diagnóstico molecular, Ensaio TaqMan®*,* Largo espectro, Clades filogenéticas

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## List of Abbreviations

AG/HE – Arthrogryposis and hydranencephaly
AINOV – Aino virus
AKAV – *Akabane orthobunyavirus*
BEFV – *Bovine fever ephemerovirus*
BHK – Baby hamster kidney
BLAST – Basic Local Alignment Search Tool
BoHV-1 – *Bovine alphaherpesvirus 1*
BTV – *Bluetongue virus*
BVDV-1 – *Pestivirus A*
CF – Complement fixation
CNS – Central nervous system
CPE – Cytophatic effect
$C_q$ – Quantification cycle
CQV – Cat Que virus
CV – Coefficient of Variation
dNTP – Deoxyribonucleotide phosphate
DOUV – Douglas virus
dsDNA – Double-stranded deoxyribonucleic acid
DVTD – Departement of Veterinary Tropical Diseases
ELISA – Enzyme-linked immunosorbent assay
FBS – Foetal bovine serum
GIS – Geographic Information System
HI – Hemagglutination inhibition
ICTV – International Committee on Taxonomy of Viruses
INGV – Ingwavuma virus
IQTV – Iquitos virus
JATV – Jatobal virus
KZN – KwaZulu-Natal
LOD – Limit of Detection
MDDV – Madre de Dios virus
MEM – Minimum essential medium
MGB – Minor groove binder
MM – Master mix
mRNA – Messenger ribonucleic acid
N – Nucleocpasid
NFQ – Non-fluorescent quencher
NS – Non-structural
ORF – Open reading frame
OROV – *Oropouche orthobunyavirus*

OYAV – Oya virus
PALV – *Palyam virus*
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
qPCR – Quantitative polymerase chain reaction
RdRp – RNA-dependent RNA polymerase
RNA – Ribonucleic acid
RSA – Republic of South Africa
RT-PCR – Reverse transcripton polymerase chain reaction
RVFV – *Rift Valley fever phlebovirus*
SABOV – Sabo virus
SATV – *Sathuperi orthobunyavirus*
SBV – Schamallenberg virus
SHAV – *Shamonda orthobunyavirus*
SHUV – *Shuni orthobunyavirus*
SIMV – *Simbu orthobunyavirus*
SNT – Serum neutralisation test
TAE – Tris-acetate-EDTA
$TCID_{50}$ – 50% Tissue Culture Infectious Dose
$T_M$ – Melting temperature
UTR – Untranslated region
WSLV – *Wesselsbron virus*

# 1. Introductory Note

## 1.1. The Internship

The final mandatory internship for the acquisition of the Integrated Masters in Veterinary Medicine (MIMV) degree by the Faculty of Veterinary Medicine, University of Lisbon, took place at Onderstepoort, Republic of South Africa, in the internationally accredited Faculty of Veterinary Sciences of the University of Pretoria.

The internship with a duration of six months had a rotational structure and a broad spectrum on what concerns to the diverse fields of activity of the Veterinary Sciences. The first rotation, programmed by the Department of Paraclinical Sciences, Section of Public Health, included training in Food Safety, applied to the Udder Health and Milk Hygiene concepts, with visits to dairy farms, as well as to the Meat Safety concept with visits to a private abattoir and a 150 000 head of cattle feedlot. The second rotation, also in the Department of Paraclinical Sciences, took place in the Section of Pathology, and included a practical approach to the anatomopathological diagnosis in domestic and wildlife species, as well as to the discussion of cases which required histopathology for a definitive diagnosis. Posteriorly, and one of my most remarkable veterinary experiences, was a rotation in the Hluvukani Animal Clinic, located in a rural village in Mpumalanga Province with an interface with the Kruger National Park, which is accommodated in a Community Programme that aims to provide primary public and animal health care, in a One Health concept approach, and where the veterinary services are reinvented due to the lack of resources. The forth rotation was in the Clinical Section of the Department of Production Animal Studies, that provides medical and surgical services to livestock and wildlife in the Onderstepoort Veterinary Academic Hospital and surrounding communities. Alongside side these, the internship's main component was the work developed in the Department of Veterinary Tropical Diseases (DVTD), which was carried out for almost the totality of time in Onderstepoort, either in part time in conjunction with the other activities, and mainly in full time. This dissertation is based on the research project developed in the DVTD.

## 1.2. The Research Project

The research project, basis of this dissertation was carried out in the DVTD, which funded this study, providing all the facilities, laboratory equipment and reagents used during its development, optimisation and validation.

All the work described furtherly was done entirely by me, under the guidelines and supervision of the DVTD's Associate Professor Melvyn Quan, who is my supervisor for this dissertation.

# 2. Introduction

The Simbu serogroup within the genus *Orthobunyavirus* belongs to the family *Peribunyaviridae* (Adams, Lefkowitz, King, Harrach, Harrison, Knowles, Kropinski, Krupovic, Kuhn, Mushegian, Nibert, Sabanadzovic, Sanfaçon, Siddell, Simmonds, Varsani, Zerbini, Gorbalenya, & Davison, 2017) and comprises 32 recognised three-segmented negative-sense single-stranded RNA viruses (Saeed, Li, Wang, Weaver, & Barrett, 2001a; Elliott & Blakqori, 2011; Ladner, Savji, Lofts, Travassos da Rosa, Wiley, Gestole, Rosen, Guzman, Vasconcelos, Nunes, T, Lipkin, Tesh, & Palacios, 2014; Tilston-Lunel, Hughes, Acrani, da Silva, Azevedo, Rodrigues, Vasconcelos, Nunes, & Elliott, 2015). This group of arthropod-borne viruses (arboviruses) is known to cause central nervous system (CNS) disease in humans (Anderson, Spence, Downs, & Aitken, 1961; Aguilar, Barrett, Saeed, Watts, Russell, Guevara, Ampuero, Suarez, Cespedes, Montgomery, Halsey, & Kochel, 2011; Ladner *et al.*, 2014) and reproductive system and CNS disease in domestic and livestock species (Coverdale, Cybinski, & St George, 1978; Charles, 1994; Coetzer & Howell, 1998; Hoffmann, Scheuch, Hoper, Jungblut, Holsteg, Schirrmeier, Eschbaumer, Goller, Wernike, Fischer, Breithaupt, Mettenleiter, & Beer, 2012; van Eeden, Williams, Gerdes, van Wilpe, Viljoen, Swanepoel, & Venter, 2012; Hirashima, Kitahara, Kato, Shirafuji, Tanaka, & Yanase, 2017). These agents have been isolated from a wide range of wild mammals and birds (Anderson, Spence, Downs, & Aitken, 1960; McIntosh, McGillivray, & Dickinson, 1965; Calisher, Kokernot, De Moore, Boyd, Hayes, & Chappell, 1969; Reeves, Scrivani, Hardy, Roberts, & Nelson, 1970; Carey, Reuben, George, Shope, & Myers, 1971; Pajot, 1980; Seymour, Peralta, & Montgomery, 1983; Navarro, Giambalvo, Hernandez, Auguste, Tesh, Weaver, Montanez, Liria, Lima, Travassos da Rosa, da Silva, Vasconcelos, Oliveira, Vianez, & Nunes, 2016). The viruses in the serogroup have a cosmopolitan distribution and have been divided phylogenetically into two different clades, designated A, those associated with clinical disease in humans, and B, associated with abortion and teratology in ruminants, as well as neurologic disease in horses (Ladner *et al.*, 2014).

Rapid viral detection and large population screening capability provide important epidemiological data, enhance the surveillance and control of emerging, reemerging or novel diseases and consequently optimise the management in terms of public and animal health (Richman, Cleveland, Redfield, Oxman, & Wahl, 1984).

Culture-based and serologic methods play a pivotal role in the diagnostic of viral infections but can be time-consuming and technically demanding, thus limiting its usefulness when rapid diagnosis is needed, further requiring skilled and experienced personnel as well as adequate facilities to manipulate cell lines or pathogenic viruses (Souf, 2016). The use of molecular technologies based on nucleic acid amplification, such as conventional or real-time PCR, has increased to the point where it is now considered as the gold standard for viral diagnostics (Mackay, Arden, & Nitsche, 2002). In comparison with the conventional technique, although the cost of equipment and reagents are substantially higher, real-time PCR has greater

analytical sensitivity and specificity, is quicker to perform, has multiplex capacitiy and allow viral genome quantification, being the most widely used method for direct virus detection (Mackay *et al.*, 2002; Wernike & Beer, 2017).

A few laboratory techniques for the broad detection of Simbu serogroup viruses have been described, based on serology, which in fact led to its original designation as serogroup (Kinney & Calisher, 1981), or on molecular techniques. Real-time PCR assays with broad detection capacity have been described, and utilise an intercalated dye chemistry for the detection of both phylogenetic clades (Fischer, Schirrmeier, Wernike, Wegelt, Beer, & Hoffmann, 2013) or Taqman® chemistry for the detection of clade B viruses (Shirafuji, Yazaki, Shuto, Yanase, Kato, Ishikura, Sakaguchi, Suzuki, & Yamakawa, 2015).

In this dissertation, a novel real-time RT-PCR assay is described, utilising Taqman® based chemistry for the broad detection of Simbu serogroup viruses, which allows for the distinction between both clades, based on fluorescence emission spectral discrimination. The development of the real-time RT-PCR assay from the evaluation of all Simbu serogroup viruses's published genomic data, in order to design specific primers and probes, until the testing of field samples, including the laboratory characterisation in terms of efficiency, sensitivity, specificity and repeatability, is described.

This work resulted in a research article that will be submitted (June 2018) for publication to the *Journal of Virological Methods*, peer-reviewed scientific journal.

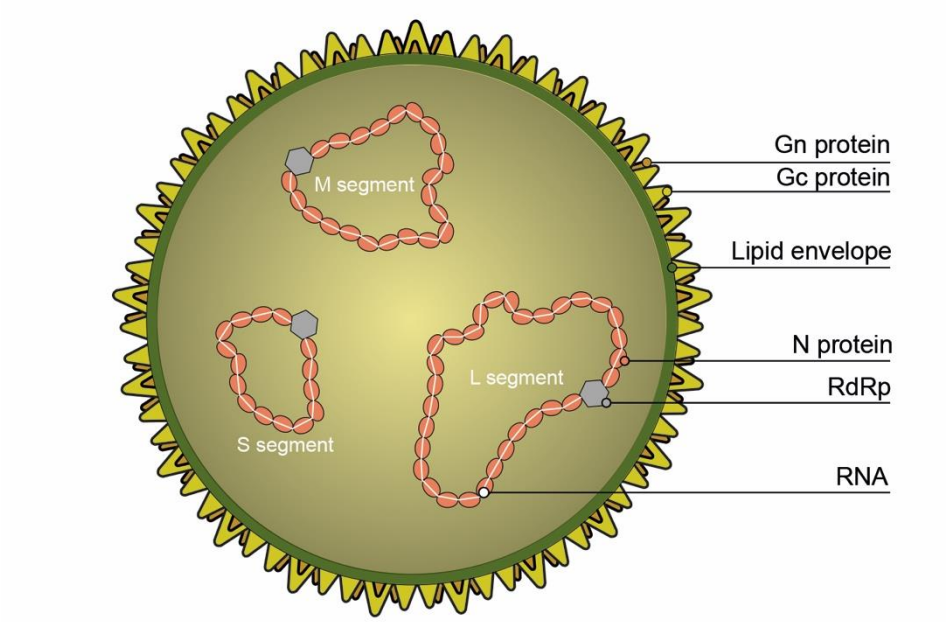
# 3. Literature Review

## 3.1. The Simbu serogroup, Genus *Orthobunyavirus*, Family *Peribunyaviridae*, Order *Bunyavirales*

### 3.1.1. General characteristics

The virions are spherical, enveloped, approximately 100 nm in diameter and have a three-segmented genome, composed of negative-sense single-stranded RNA (Elliott & Blakqori, 2011) (Figure 3.1).

**Figure 3.1. General characteristics of virions of the Order *Bunyavirales* (Original).**




The genus *Orthobunyavirus* currently belongs to the family *Peribunyaviridae* (Adams *et al.*, 2017) comprising 49 species of virus represented by approximately 170 examplar isolates, the exact number varies with sporadic discovery of new isolates or taxonomic revision of existing viruses. Historically, the viruses were classified into 18 serogroups according to their antigenic relationships in hemagglutination inhibition (HI), complement fixation (CF) and serum neutralisation tests (SNT) (Kinney & Calisher, 1981; Calisher, 1983; Travassos da Rosa, Tesh, Pinheiro, Travassos da Rosa, & Peterson, 1983; Elliott & Blakqori, 2011; Chowdhary, Street, Travassos da Rosa, Nunes, Tee, Hutchison, Vasconcelos, Tesh, Lipkin, & Briese, 2012; Gauci, McAllister, Mitchell, Boyle, Bulach, Weir, Melville, & Gubala, 2015). The Simbu serogroup is one of the largest of these serogroups and is named after the prototype virus (Saeed *et al.*, 2001a). The concept of serogroup played an important role in the development of viral taxonomy (Calisher & Karabatsos, 1988), especially for the classification of the arboviruses (Casals, 1957) although the term is no longer used by the International Committee on Taxonomy of Viruses (ICTV), as the comparative analysis of nucleic acid and protein

sequences has become the primary method for determining virus relationships (Saeed *et al.*, 2001a). However, due to the lack of genetic data for many named arboviruses, most current taxonomic assingments are still based on serology (Nichol, Beaty, Elliott, Goldbach, Plyusnin, Schmaljohn, & Tesh, 2005; Plyusnin, Beaty, Elliott, Goldbach, Kormelink, Lundkvist, Schmaljohn, & Tesh, 2012). In addition, antigenic relationships remain important in establishing serodiagnosis or in conducting serosurveys.

The Simbu serogroup consists of a highly diverse group of related arboviruses that infect not only humans, but also economically important livestock species and an extensive number of wildlife animals (Ladner *et al.*, 2014; Zhang, Wang, Wang, Fu, Li, Zhao, Zhu, Wang, & Liang, 2015). The members of the group were identified in a widely geographically distributed range of vertebrate and invertebrate hosts, from dry deserts to tropical forests (Costa Mendes, 1984).

In 1981, Kinney & Calisher published the most comprehensive comparison of the antigenic relationships among all recognised Simbu serogroup viruses. Even though the serogroup members were distinguishable by serum neutralization assays, the viruses showed extensive cross-reactions in the CF assays. On the basis of CF cross reactivity and subsequent difficulty to distinguish viruses from each other, the authors proposed the allocation of the 24 viruses into five complexes named after the first isolated member of each complex: the Simbu complex (Simbu, Akabane, Yaba-7, Shamonda, Sabo, Tinaroo, Sango, Peaton, Aino and Sathuperi viruses), the Manzanilla complex (Manzanilla, Ingwavuma, Mermet, Inini and Buttonwillow viruses) and the Oropouche complex (Oropouche, Utinga, Utive and Facey's Paddock viruses), plus Thimiri and Nola viruses which were distinct in the CF test and therefore designated as the sole members of their complexes (Kinney & Calisher, 1981).

Almost thirty years later, the Simbu serogroup included 25 antigenically related viruses, by then classified into seven different complexes, including Simbu, Manzanilla, Oropouche, Akabane, Sathuperi, Shamonda and Shuni (Yanase, Kato, Aizawa, Shuto, Shirafuji, Yamakawa, & Tsuda, 2012).

At present, the Simbu serogroup has 32 recognised members, of which 28 have been subjected to full genome sequencing (Table 3.1) (Saeed *et al.*, 2001a; Ladner *et al.*, 2014; Tilston-Lunel *et al.*, 2015; Zhang *et al.*, 2015). The viruses fall into two phylogenetic clades, designated A and B. Clade A comprise mainly viruses that occur in the New World (but including Facey's Paddock virus from Australia, Cat Que virus from Asia, and Ingwavuma virus that occurs in Africa and Asia) and clade B members are distributed widely in Africa, Asia and Oceania, with a few individual viruses occurring in all three regions. The recent discovery of Schmallenberg virus (SBV) extends the known distribution of the clade into Europe (Table 3.2). Leanyer virus (LEAV), of Australia, does not fall into either of the two clades. The 31 viruses of clades A and B have recently been assigned to 8 species on the basis of close antigenic relationships and the existence of less than 10% differences in amino acid sequences of the nucleocapsid protein gene between the members of each species (Table 3.2) (ICTV, 2015).

**Table 3.1. Known and potential members of the Simbu serogroup orthobunyaviruses, showing the origins of the prototype isolates (Swanepoel & Camarão, 2017).**

| | Virus | ICTV Acronym | Prototype isolate | Year | Country | Source | Putative vectors | Known distribution |
|---|---|---|---|---|---|---|---|---|
| 1 | Manzanilla | MANV | TRVL 3587 | 1954 | Trinidad | Howler monkey (*Alouatta insularis*) | Mosquitoes, midges | Central America |
| 2 | Simbu | SIMV | SA Ar 53 | 1955 | South Africa | Mosquitoes | Mosquitoes, midges | Africa |
| 3 | Oropouche | OROV | TRVL 9760 | 1955 | Trinidad | Human | Mosquitoes, midges | Central/South America |
| 4 | Sathuperi | SATV | IG 10310 | 1957 | India | Mosquitoes | Mosquitoes | Asia, Africa |
| 5 | Ingwavuma | INGV | SA An 4165 | 1959 | South Africa | Spectacled Weaver *(Hyphanturgus ocularius)* | Mosquitoes, midges | Africa, Asia |
| 6 | Akabane | AKAV | Ja Gar 39 | 1959 | Japan | Mosquitoes | Midges | Asia, Africa, Australia |
| 7 | Buttonwillow | BUTV | A 7956 | 1962 | USA | Cottontail rabbit (*Sylvilagus auduboni)* | Mosquitoes, midges | North America |
| 8 | Yaba-7 | Y7V | Y-7 | 1963 | Nigeria | *Culicoides* midges | | Africa |
| 9 | Thimiri | THIV | VRC 66414 | 1963 | India | Indian Pond Heron (*Ardeola grayii*) | | Asia |
| 10 | Aino | AINOV | JaNAr 28 | 1964 | Japan | Mosquitoes | Mosquitoes, midges | Asia, Australia |
| 11 | Mermet | MERV | AV-782 | 1964 | USA | Purple martin (*Progne subis*) | Mosquitoes | North America |
| 12 | Utinga | UTIV | BeAn 84785 | 1965 | Brazil | Brown-throated sloth (*Bradypus trydactylus*) | | South America |
| 13 | Sango | SANV | An 5077 | 1965 | Nigeria | Bovine | Mosquitoes, midges | Africa |
| 14 | Shamonda | SHAV | An 5550 | 1965 | Nigeria | Bovine | Midges | Africa |
| 15 | Shuni | SHUV | An 10107 | 1966 | Nigeria | Bovine | Mosquitoes, midges | Africa |
| 16 | Sabo | SABOV | AN 9398 | 1966 | Nigeria | Caprine | Midges | Africa |
| 17 | Kaikalur | KAIV | VRC7 13423-2 | 1971 | India | Mosquitoes | Mosquitoes | Asia |
| 18 | Inini | INIV | CaAn-128d | 1973 | French Guiana | Aracari bird (*Pteroglossus aracari*) | | South America |
| 19 | Facey's Paddock | FPV | Aus Ch 16129 | 1974 | Australia | Mosquitoes | | Australia |
| 20 | Leanyer | LEAV | NT 16701 | 1974 | Australia | Mosquitoes | Mosquitoes | Australia |
| 21 | Utive | UVV | Pan An 48878 | 1975 | Panama | Pale-throated sloth (*Bradypus variegatus*) | | Central America |
| 22 | Pintupo | Pending | PanAr 517 | 1976? | Panama | *Culicoides* midges | Midges | Central America |
| 23 | Peaton | PEAV | CSIRO 110 | 1976 | Australia | *Culicoides* midges | Midges | Australia |
| 24 | Douglas | DOUV | CSIRO 150 | 1978 | Australia | Bovine | Midges | Australia, New Guinea |
| 25 | Tinaroo | TINV | CSIRO 153 | 1978 | Australia | Culicoides midges | Midges | Australia |
| 26 | Jatobal | JATV | BeAn 423380 | 1985 | Brazil | Coati (*Nasua nasua*) | Mosquitoes, midges | South America |
| 27 | Iquitos | IQTV | IQT9924 | 1999 | Peru | Human | Midges | South America |
| 28 | Oya | OYAV | | 1999 | Malaysia | Pig | | Asia |
| 29 | Cat Que | Pending | VN 04-2108 | 2004 | Vietnam | Mosquitoes | Mosquitoes | Asia |
| 30 | Madre de Dios | MDDV | FMD 1303 | 2007 | Peru | Human | Mosquitoes, midges? | South America |
| 31 | Schmallenberg | SBV | BH80/11-4 | 2011 | Germany | Bovine | Midges | Europe |
| 32 | Perdões | Pending | BeAn 790177 | 2012 | Brazil | Black-tufted marmoset (*Callitrhix penicillata*) | | South America |

**Table 3.2. Phylogeny of members of the Simbu serogroup orthobunyaviruses (Ladner *et al.*, 2014; ICTV, 2015).**

| Species | Virus | Distribution |
|---|---|---|
| **CLADE A** | | |
| ***Oropouche orthobunyavirus*** | Oropouche | Central/South America |
| | Facey's Paddock | Australia |
| | Iquitos | South America |
| | Jatobal | South America |
| | Madre de Dios | South America |
| | Perdões | South America |
| | Pintupo | Central America |
| | Utinga | South America |
| | Utive | Central America |
| ***Manzanilla orthobunyavirus*** | Manzanilla | Central America |
| | Buttonwillow | North America |
| | Oya | Asia |
| | Cat Que | Asia |
| | Ingwavuma | Africa, Asia |
| | Inini | South America |
| | Mermet | North America |
| **CLADE B** | | |
| ***Akabane orthobunyavirus*** | Akabane | Asia, Africa, Australia |
| | Tinaroo | Australia |
| | Sabo | Africa |
| | Yaba-7 | Africa |
| ***Sathuperi orthobunyavirus*** | Sathuperi | Asia, Africa |
| | Douglas | Australia, New Guinea |
| | Schmallenberg | Europe |
| ***Shamonda orthobunyavirus*** | Shamonda | Africa |
| | Sango | Africa |
| | Peaton | Australia |
| ***Simbu orthobunyavirus*** | Simbu | Africa |
| ***Shuni orthobunyavirus*** | Shuni | Africa |
| | Aino | Asia, Australia |
| | Kaikalur | Asia |
| ***Thimiri orthobunyavirus*** | Thimiri | Asia |
| **UNGROUPED** | | |
| ***Leanyer orthobunyavirus*?** | Leanyer | Australia |

*Manzanilla orthobunyavirus* (MANV), the first to be mentioned in the literature, was recovered in Trinidad and Tobago, in 1954, from the blood of a howler monkey (*Alouatta seniculus insularis*) (Anderson *et al.*, 1960). Recently, MANV was isolated from pools of *Culex tritaeniorhynchus* in Yunnan Province, People's Replubic of China (Feng, Fu, Yang, Zhang, He, Tu, Liang, & Zhang, 2015).

*Simbu orthobunyavirus* (SIMV) was isolated originally in the Republic of South Africa (RSA) from *Aedes circumluteolus* mosquitoes caught between 1955 and 1957, at Lake Simbu, Northern KwaZulu-Natal (KZN) (Weinbren, Heymann, Kokernot, & Paterson, 1957; Brooke Worth, Paterson, & De Meillon, 1961). SIMV was isolated subsequently in Cameroon, in 1966, from a pool of *Eretmapodites chrysogaster* mosquitoes (Salaun, Rickenbach, Bres, Brottes, Germain, Eouzan, & Ferrara, 1969). Human seroconversion was recorded in RSA and Botswana (Fraenkel-Conrat & Wagner, 1979).

*Oropouche orthobunyavirus* (OROV) was first isolated from the blood of a febrile forest worker in a village called Vega de Oropouche in 1955, and then from a pool of *Coquillettidia venezuelensis* mosquitoes in the Bush Bush Forest in Trinidad and Tobago, in 1960 (Anderson *et al.*, 1961). In Brazil, OROV has been isolated from vertebrates such as humans, the three-toed sloth (Bradypus *tridactylus*), and the marmoset (*Callithrix sp.)*, as well as from arthropods such as *Aedes (Ochlerotatus) serratus, Culex fatigans, C. pipiens quinquefasciatus* mosquitoes and *Culicoides paraensis* midges (Pinheiro, Pinheiro, Bensabath, Causey, & Shope, 1962; Pinheiro, Bensabath, Andrade, & Woodal 1968; Pinheiro, Travassos da Rosa, Travassos da Rosa, & Bensabath, 1976; Pinheiro, Hoch, Gomes, & Roberts, 1981; Nunes, Martins, Rodrigues, Chiang, Azevedo Rdo, da Rosa, & Vasconcelos, 2005). In 1989, the presence of OROV was reported in the village of Bejuco in Panama (Pinheiro, Travassos da Rosa, & Vasconcelos, 2004) and between 1992 and 1994, the pathogen was isolated in the Amazon region of Peru, in the cities of Iquitos, Puerto Maldonado e Madre de Dios (Watts, Lavera, Callahan, Rossi, Oberste, Roehrig, Cropp, Karabatsos, Smith, Gubler, Wooster, Nelson, & Hayes, 1997; Baisley, Watts, Munstermann, & Wilson, 1998). OROV exists as three different genotypes, namely I (Trinidad and Brazil), II (Brazil and Peru), and III (Panama and Brazil) (Saeed, Wang, Nunes, Vasconcelos, Weaver, Shope, Watts, Tesh, & Barrett, 2000). The true public health implication of OROV infection in South America remains unclear, due to the clinical resemblance of OROV infection to other endemic arboviral diseases such as Dengue, Mayaro, Chikungunya or Zika fevers, and the paucity of cases confirmed by laboratorial diagnosis (Navarro *et al.*, 2016). Since the first isolation of the virus in 1955, it has been estimated to have caused more than 30 outbreaks and affected more than half a million people, which would make it one of the most common arboviruses affecting humans in tropical America (Travassos da Rosa, de Souza, Pinheiro, Figueiredo, Cardoso, Acrani, & Nunes, 2017). The clinical presentation of an affected human is an acute febrile illness, usually accompanied by headache, myalgia, arthralgia, anorexia, dizziness, chills, photophobia, nausea, vomiting, diarrhoea, conjunctive congestion, epigastric pain, retro-orbital pain and other systemic manifestations (Pinheiro, Travassos da Rosa, Travassos da Rosa, Ishak, Freitas, Gomes, LeDuc, & Oliva, 1981).

*Sathuperi orthobunyavirus* (SATV), recovered originally in India from pools of *Culex vishnui* mosquitoes (Dandawate, Rajagopalan, Pavri, & Work, 1969) was identified thereafter in Nigeria in dairy cattle and pools of *Culicoides* spp. (Lee, 1979). In 1999, two viruses closely related to the ones described previously were isolated from sentinel cattle in the Okayama Prefecture of Japan (Yanase, Fukutomi, Yoshida, Kato, Ohashi, Yamakawa, & Tsuda, 2004).

Ingwavuma virus (INGV), isolated originally in South Africa, in 1959, from the organs of a spectacled weaver (*Hyphanturgus ocularius*) and from a pool of *Culex univittatus* mosquitoes (McIntosh *et al.*, 1965), was *a posteriori* recovered from the whole blood of an Indian pond heron (*Ardeola grayii*) in India (originally referred to as Bagalodu virus) and from a spotted

flycatcher (*Muscicapa striata*) in Cyprus (Pavri, 1969; Watson, 1969). In Thailand, the virus was isolated from swine whole blood and from pools of *Culex vishnui* mosquitoes (Top, Kraivapan, Grossman, Rozmiarek, Edelman, & Gould, 1974) and in 1985, in Indonesia, was recovered from *Culex* spp. and *Mansonia* spp. mosquitoes (Converse, Tan, Rachman, Lee, & Shope, 1985).

*Akabane orthobunyavirus (*AKAV*)*, was recognised originally in the Gunma Prefecture of Japan in pools of *Aedes vexans* as well as *Culex tritaeniorhynchus* mosquitoes in 1959 (Matsuyama, Oya, Ogata, Kobayashi, Nakamura, Takahashi, & Kitaoka, 1960). In 1968, the virus was isolated from pools of *Culicoides brevitarsis* in Australia (Doherty, Carley, Standfast, Dyce, & Snowdon, 1972) and in 1972, was recovered in Kenya from pools of *Anopheles funestus* (Metselaar & Robin, 1976). The virus was described as a natural infection in sentinel flocks of ewes in Australia, in 1976, in which the virus was isolated (Della-Porta, O'Halloran, Parsonson, Snowdon, Murray, Hartley, & Haughey, 1977). Serological evidence suggests that AKAV occurs in the Middle East region, namely in the Turkish Province of Aydin, Cyprus and Orontes river valley of Syria (Taylor & Mellor, 1994). Neutralising antibodies were found in a wide range of domestic animals in the Arabian peninsula, particularly in Kuwait, Saudi Arabia and Bahrain (Al-Busaidy, Mellor, & Taylor, 1988) and in a considerable number of African wild species, such as buffalo, nyala, bushbuck, kudu, eland, waterbuck, reedbuck, lechwe, roan and sable antelope, gemsbok, tsessebe, topi, blesbok, red hartebeest, blue wildebeest, impala, springbok, oribi, hippopotamus, giraffe, bushpig, warthog and elephant (Al-Busaidy, Hamblin, & Taylor, 1987). In Japan, after 1959, the pathogen was isolated a few times between 1974 and 2003, mainly in bovine samples and *Culicoides* spp. (Yamakawa, Yanase, Kato, & Tsuda, 2006). AKAV was also recovered from bovines and swines in Taiwan (Liao, Lu, Goto, & Inaba, 1996; Chang, Liao, Su, Farh, & Shiuan, 1998; Huang, Huang, Deng, Jong, & Lin, 2003), from mosquitoes in the Yunnan Province of China (Feng, He, Fu, Yang, Zhang, Tu, Liang, & Zhang, 2015) and from cattle in South Korea (Bak, Lim, Cheong, Hwang, & Cho, 1980; Oem, Lee, Kim, Bae, Chung, Lee, & Roh, 2012). Substantial seroprevalence in cattle, sheep and goats was also reported in China (Wang, Blasdell, Yin, & Walker, 2017). In 1969-70, an outbreak of Akabane disease in domestic ruminants occurred in Israel (Shimshony, 1980), and again between 2001 and 2003 (Brenner, Tsuda, Yadin, Chai, Stram, & Kato, 2004), where a new lineage was described (Stram, Brenner, Braverman, Banet-Noach, Kuznetzova, & Ginni, 2004a). AKAV can cause abortion, stillbirth, congenital malformations, principally arthrogryposis (AG) and hydranencephaly (HE) (congenital AG/HE syndrome), as well as marked teratology, particularly of the CNS, if the virus infects the foetus at a critical stage of development and when susceptible pregnant ruminants are infected. In non-pregnant animals the infection is subclinical (St. George & Kirkland, 2004). The stage of gestation in which the host is infected determines the range and severity of clinical signs (Uchida, Murakami, Sueyoshi, Tsuda, Inai, Acorda, Yamaguchi, & Tateyama, 2000). Bovine and swine cases of

AKAV encephalomyelitis have also been described (Miyazato, Miura, Hase, Kubo, Goto, & Kono, 1989; Uchida *et al.*, 2000; Kono, Hirata, Kaji, Goto, Ikeda, Yanase, Kato, Tanaka, Tsutsui, Imada, & Yamakawa, 2008; Oem *et al.*, 2012). Recently, a congenital AG/HE syndrome in newborn calves was reported in Iraq (Alsaad, Alautaish, & Alamery, 2017).

Buttonwillow virus (BUTV), the first isolate in Northern America was identified originally in whole blood of a desert cottontail rabbit (*Sylvilagus audubonii)* and later in a blacktail jackrabbit (*Lepus californicus*) and pools of *Culicoides variipennis* midges. Seroconversion was described in lagomorphs (*S. audubonii, L. californicus*, *L. americanus*) across USA and in one Canadian marmot (Reeves *et al.*, 1970; Nelson & Scrivani, 1972).

The first Simbu serogroup virus recovered in Nigeria, in Yaba, a suburb of Lagos, was named Yaba-7 virus (Y7V), and was isolated from a pool of *Mansonia africana* mosquitoes in 1963 (Theiler & Downs, 1973).

*Thimiri orthobunyavirus* (THIV), like INGV, was isolated for the first time from the whole blood of an Indian pond heron (*Ardeola grayii*) in India, in 1963 (Carey *et al.*, 1971), and subsequently in Egypt, between 1963 and 1966, in the blood of migratory birds, namely a common (*Sylvia communis*) and a lesser whitethroat (*S. curruca*) (Darwish & Hoogstraal, 1981) and in the north of Australia from a pool of *Culicoides histrio* bird-feeding midges (Standfast & Dyce, 1982).

Aino virus (AINOV) was identified originally in Nagasaki, Japan, in pools of *Culex tritaeniorhynchus* and in a mixed pool of *C. pseudovishnui* and *C. pipiens* mosquitoes (Takahashi, Oya, Okazda, Matsuo, & Kuma, 1968) and then isolated in Australia from pools of *Culicoides brevitarsis* (Doherty *et al.*, 1972). In 2003, serological evidence of its presence in dairy herds in Israel was recorded (Brenner *et al.*, 2004). Clinical cases of congenital AG/HE syndrome showed high titres of neutralising antibodies to AINOV (Miura, Hayashi, Ishihara, Inaba, & Omori, 1974; Coverdale *et al.*, 1978) and experimentally AINOV caused the same syndrome associated with cerebellar hypoplasia in calves (Tsuda, Yoshida, Ohashi, Yanase, Sueyoshi, Kamimura, Misumi, Hamana, Sakamoto, & Yamakawa, 2004).

The second Simbu serogroup member to be isolated in North America, Mermet virus (MERV) was recovered originally from the whole blood of a purple martin (*Progne subis*) in Metropolis, Illinois, in 1964. MERV was also recovered from other birds, namely a red-winged blackbird (*Agelaius phoeniceus*), a Swainson's thrush (*Catharus ustulatus*) and a cardinal (*Cardinalidae*) in the Ohio-Mississipi basin (Calisher *et al.*, 1969). Another strain was obtained from a mixed pool of *Culex pipiens* and *C. restuans* mosquitoes in Memphis, Tennessee (Jakob, Francy, Trimble, & Calisher, 1979). In 1969, during an ecologic investigation of an outbreak of Venezuelan equine encephalitis (VEE) in Guatemala, MERV was isolated from an Altamira oriole (*Icterus gularis*) (Gutierrez, Calisher, Maness, & Lord, 1975).

Utinga virus (UTIV), was isolated originally in 1965 from the whole blood and pooled organs of a pale-throated sloth (*Bradypus tridactylus)* in Brazil (Karabatsos, 1985) and no further isolations have been published in the literature.

Sango virus (SANV), the second Nigerian Simbu serogroup isolate to be recovered, was isolated in bovine whole blood and in pools of *Culicoides* spp. during a surveillance study by the Virus Research Laboratory of the University of Ibadan (Causey, Kemp, Madbouly, & Lee, 1969; Causey, Kemp, Causey, & Lee, 1972; Lee, 1979). It was later recovered from pools of *Mansonia uniformis* in Kenya (Metselaar, Henderson, Kirya, Tukei, & de Geus, 1974) and seroconversion against SANV was reported in dairy cattle and goats in Ibadan (Kemp, Causey, & Causey, 1971).

The third Nigerian Simbu serogroup to be isolated, *Shamonda orthobunyavirus* (SHAV), was first identified in bovine whole blood during the same surveillance study mentioned previously (Causey *et al.*, 1969; Causey *et al.*, 1972) and in *Culicoides imicola* pools (Lee, 1979) collected and caught respectively in Ibadan. Antibodies against SHAV were also found in cattle in Ibadan (Kemp *et al.*, 1971). In 1967, in Vryburg, South Africa, SHAV was associated with an outbreak of disease in cattle characterised by conjunctival and periorbital oedema as well as swollen lips and tongue, although the pathogen was named Lambrecht virus originally (Solberg, 1970; Costa Mendes, 1984). In the same year and country, the virus was recovered from *Culicoides imicola* midges caught at the Onderstepoort Veterinary Institute (OVI) facilities (McIntosh, 1980). In 2002, serological and molecular evidence of SHAV was reported in the Kagoshima and Miyazaki prefectures of Japan (Yanase, Maeda, Kato, Nyuta, Kamata, Yamakawa, & Tsuda, 2005). Recently, in southern Japan, SHAV was identified in association with congenital malformations, including torticollis, arthrogryposis, spinal curvature, ventriculomegaly and cerebellar hypoplasia, in calves infected *in utero* (Hirashima *et al.*, 2017).

*Shuni orthobunyavirus* (SHUV), fourth isolate to be described in Nigeria, was isolated originally from a cow in Sokoto (Causey *et al.*, 1969), from the whole blood of a sheep in Ibadan (Causey *et al.*, 1972), from cattle in northern Nigeria (Kemp, Causey, Moore, & O'Connor, 1973) and from pools of *Culicoides* spp. (Lee, 1979). The presence of neutralising antibodies in cattle and sheep was reported in the same country (Kemp *et al.*, 1971). In 1975, Moore *et al.* described the isolation of SHUV from the blood of a Nigerian child (Moore, Causey, Carey, Reddy, Cooke, Akinkugbe, David-West, & Kemp, 1975). In South Africa, the virus was isolated in *Culex theileri* mosquitoes in Gauteng and in ruminants in KZN (McIntosh, 1972, 1980), from the brain of two horses with CNS clinical signs in Zimbabwe (Foggin & Swanepoel, 1990) and in RSA (Coetzer & Howell, 1998). More recently, the role of SHUV in neurological disease in horses was further reported (van Eeden *et al.*, 2012) and, interestingly, the seroconversion against SHUV in veterinary professionals in South Africa (van Eeden, Swanepoel, & Venter, 2014a). Between 2014-15, the role of SHUV in malformed and aborted foetus of small

ruminants and cattle in Israel was investigated (Golender, Brenner, Valdman, Khinich, Bumbarov, Panshin, Edery, Pismanik, & Behar, 2015).

Sabo virus (SABOV), was isolated in Ibadan, Nigeria during the surveillance study mentioned previously (Causey *et al.*, 1969; Kemp *et al.*, 1971; Causey *et al.*, 1972; Lee, 1979), from a goat, cattle and *Culicoides* spp.. Neutralising antibodies were found in cattle, small ruminants and pigs.

Kaikalur virus (KAIV) was isolated from a pool of *Culex tritaeniorhynchus* mosquitoes caught in 1971, in Kaikalur, Andhra Pradesh, India (Rodrigues, Singh, Dandawate, Soman, & Bhatt, 1977).

Inini virus (INIV), was recovered from a bank of a river with the same name, from the whole blood of a Aracari bird (*Pteroglossus aracari*), in Maripasoula, French Guiana (Pajot, 1980).

Facey's Paddock virus (FPV), was identified originally in pools of *Culex annulirastris, Aedes narmanensis* and *Culicoides* spp. in Australia. The presence of neutralising antibodies was reported in cattle and wallabies (Doherty *et al.*, 1972; Doherty, 1975; Doherty, Carley, Kay, Filippich, Marks, & Frazier, 1979).

Leanyer virus (LEAV) was isolated from *Anopheles meraukensis* mosquitoes collected at Leanyer, in the Northern Territory of Australia, in 1974, during an entomological surveillance in the course of an outbreak of Murray Valley encephalitis (MVE) (Doherty, Carley, Filippich, Kay, Gorman, & Rajapaksa, 1977).

Utive virus (UVV) was isolated in Panama, in a village with the same name, from the blood of a brown-throated sloth (*Bradypus variegatus*) in 1975 (Seymour *et al.*, 1983).

In Panama, Pintupo virus, was isolated from *Culicoides diabolicus* (Seymour *et al.*, 1983).

Another Australian isolate, Peaton virus (PEAV), was recovered originally from pools of *Culicoides brevitarsis* and bovine whole blood (St George, Cybinski, Filippich, & Carley, 1979; St George, Standfast, Cybinski, Filippich, & Carley, 1980). In 1999, the virus was identified by SNT in *Culicoides* midges and cattle blood in the Kyushu district of Japan (Matsumori, Inai, Yanase, Ohashi, Kato, Yoshida, & Tsuda, 2002). The association of PEAV with congenital malformations in sheep via experimental infection has been described (Parsonson & McPhee, 1985).

Douglas virus (DOUV) was identified in the blood of a cow with no clinical signs, and thereafter in pools of *Culicoides brevitarsis* in Australia. In the same country, neutralising antibodies in all the species of domestic ruminants, as well as in buffalo and deer were found. In Papua New Guinea, a bovine that seroconverted against DOUV was identified (Doherty *et al.*, 1979; Cybinski, 1984).

Tinaroo virus (TINV) was identified first in pools of *Culicoides brevitarsis* and later in the whole blood of cattle in Australia. In the same country, a lamb with AG syndrome had neutralising

antibodies against TINV, and similar to DOUV, antibodies were found in cattle, sheep, goat, buffalo and deer (Doherty *et al.*, 1979; Cybinski, 1984).

In 1985, Jatobal virus (JATV) was recovered from the whole blood of a coati (*Nasua nasua)* in Tucuruí, Pará State, Brazil and classified as a distinct member of the serogroup (Figueiredo & Da Rosa, 1988). Sixteen years after, it was suggested that JATV was a possible reassortant of OROV (Saeed, Wang, Suderman, Beasley, Travassos da Rosa, Li, Shope, Tesh, & Barrett, 2001b).

Iquitos virus (IQTV), was isolated in a city with the same name in Peru, from a thirteen-year-old boy with a clinical presentation of fever, headache, eye and body pain, arthralgia, diarrhea, and chills, in 1999. IQTV was also described as a reassortant of OROV (Aguilar *et al.*, 2011).

In Malaysia, Oya virus (OYAV) was isolated from a pig suspected of Nipah virus (NiV) infection, during a national swine surveillance programme, and named after the Oya village from where it was recovered. Interestingly 93% (n=360) seropositivity was observed in a serosurvey of pigs in the principal Malaysian pig breeding districts (Kono, Yusnita, Mohd Ali, Maizan, Sharifah, Fauzia, Kubo, & Aziz, 2002). Recently, a retrospective study reported the presence of viral RNA in pig and human samples, as well as immunoglobulin G (IgG) positivity in human serum in the Karnataka State of India (Yadav, Shete, Bondre, Patil, Kokate, Chaudhari, Srivastava, Jadhav, & Mourya, 2016).

Cat Que virus (CQV) was isolated from mosquitoes during an arbovirus surveillance in an outbreak of acute paediatric encephalitis in northern Vietnam in 2004, in spite of the virus reported originally to be OYAV, based on indirect immunofluorescence assays and high nucleotide similarity (Bryant, Crabtree, Nam, Yen, Duc, & Miller, 2005). Consequent analysis showed 4,1% amino acid and 9,6% nucleotide divergence from the original OYAV isolate (Ladner *et al.*, 2014). In 2015, the isolation of CQV from *Culex tritaeniorhynchus* mosquitoes in the Sichuan Province of China as well as anti-CQV IgM/IgG in pigs was reported (Zhang *et al.*, 2015).

Madre de Dios virus (MDDV), is an unofficial name proposed to refer to an isolate recovered in 2007 from a febrile human in Madre de Dios, region in the southeastern Peru's Amazon basin. MDDV was also described as a OROV reassortant, identical at the amino acid level to both OROV and IQTV but due to its distinctiveness was suggested that the pathogen should have its own name (Ladner *et al.*, 2014). In 2010, MDDV was obtained from a wedge-capped capuchin (*Cebus olivaceus*), collected in Atapirire village, located in the southeastern part of Venezuela, during an epizootic of illness among sylvatic monkeys (Navarro *et al.*, 2016).

In the autumn of 2011, an unidentified disease in dairy cattle with a clinical presentation of fever, diarrhoea, and decreased milk production, was reported to the veterinary services, local diagnostic laboratories, and national research institutes in Germany and the Netherlands (Hoffmann *et al.*, 2012). After excluding endemic and emerging diseases such as infectious

bovine rhinotracheitis (BoHV-1), bovine viral diarrhoea (BVDV-1), bluetonge (BTV), epizootic haemorrhagic disease (EHDV), foot-and-mouth disease (FMDV), Rift Valley fever (RVFV) or bovine ephemeral fever (BEFV), the blood samples from acutely diseased cows were subjected to a metagenomic approach and next-generation sequencing (NGS) to identify a novel *Orthobunyavirus* that was named after the origin of the samples as Schmallenberg virus (SBV) (Hoffmann *et al.*, 2012). SBV was the first virus of the Simbu serogroup to be reported in Europe (Saeed *et al.*, 2001a; Hoffmann *et al.*, 2012) and spread very rapidly throughout the domestic and wild ruminant population of the continent. Virus isolation or seroconversion were reported in France, Belgium, Luxembourg, Italy, Spain, United Kingdom, Ireland, Poland, Lithuania, Latvia, Switzerland, Austria, Denmark, Sweden, Norway, Finland, Czech Republic, Croatia, Slovenia, Estonia, Hungary, Greece and Portugal (Bradshaw, Mooney, Ross, Furphy, O'Donovan, Sanchez, Gomez-Parada, & Toolan, 2012; EFSA, 2012; European Food Safety, 2012; Garigliany, Bayrou, Kleijnen, Cassart, & Desmecht, 2012; Garigliany, Hoffmann, Dive, Sartelet, Bayrou, Cassart, Beer, & Desmecht, 2012; Rasmussen, Kristensen, Kirkeby, Rasmussen, Belsham, Bodker, & Botner, 2012; Bouwstra, Kooi, de Kluijver, Verstraten, Bongers, van Maanen, Wellenberg, van der Spek, & van der Poel, 2013; Conraths, Peters, & Beer, 2013; EFSA, 2013; Larska, Krzysiak, Smreczak, Polak, & Zmudzinski, 2013; Larska, Polak, Grochowska, Lechowski, Zwiazek, & Zmudzinski, 2013; Mason, Stevenson, Carty, Hosie, Caldow, & Boyes, 2013; Balmer, Vögtlin, Thür, Büchi, Abril, Houmard, Danuser, & Schwermer, 2014; Chaintoutis, Kiossis, Giadinis, Brozos, Sailleau, Viarouge, Breard, Papanastassopoulou, Zientara, Papadopoulos, & Dovas, 2014; Rasmussen, Kirkeby, Bodker, Kristensen, Rasmussen, Belsham, & Botner, 2014; Steinrigl, Schiefer, Schleicher, Peinhopf, Wodak, Bago, & Schmoll, 2014; Wernike, Conraths, Zanella, Granzow, Gache, Schirrmeier, Valas, Staubach, Marianneau, Kraatz, Höreth-Böntgen, Reimann, Zientara, & Beer, 2014; Wisloff, Nordvik, Sviland, & Tonnessen, 2014; Xavier, Joan, Roser, Rosa, Jorge, Ignasi, Marti, Joaquim, Santiago, & Oscar, 2014; Lazutka, Spakova, Sereika, Lelesius, Sasnauskas, & Petraityte-Burneikiene, 2015; Esteves, Mesquita, Vala, Abreu-Silva, van der Poel, & Nascimento, 2016; Laloy, Braud, Breard, Kaandorp, Bourgeois, Kohl, Meyer, Sailleau, Viarouge, Zientara, & Chai, 2016). The pathogenesis of SBV results in congenital malformations, premature birth or stillbirth, or mummification, when naïve dams are infected during a critical phase of pregnancy (Wernike, Elbers, & Beer, 2015). Interestingly, anti-SBV antibodies were found in dogs in Sweden and France and viral RNA was isolated from the brain of a puppy with neurologic signs (Sailleau, Boogaerts, Meyrueix, Laloy, Bréard, Viarouge, Desprat, Vitour, Doceul, Boucher, Zientara, Nicolier, & Grandjean, 2013; Wensman, Blomqvist, Hjort, & Holst, 2013). Full genome investigation has shown that SBV belongs to the species *Sathuperi orthobunyavirus* and is a possible ancestor of SHAV (Goller, Hoper, Schirrmeier, Mettenleiter, & Beer, 2012).

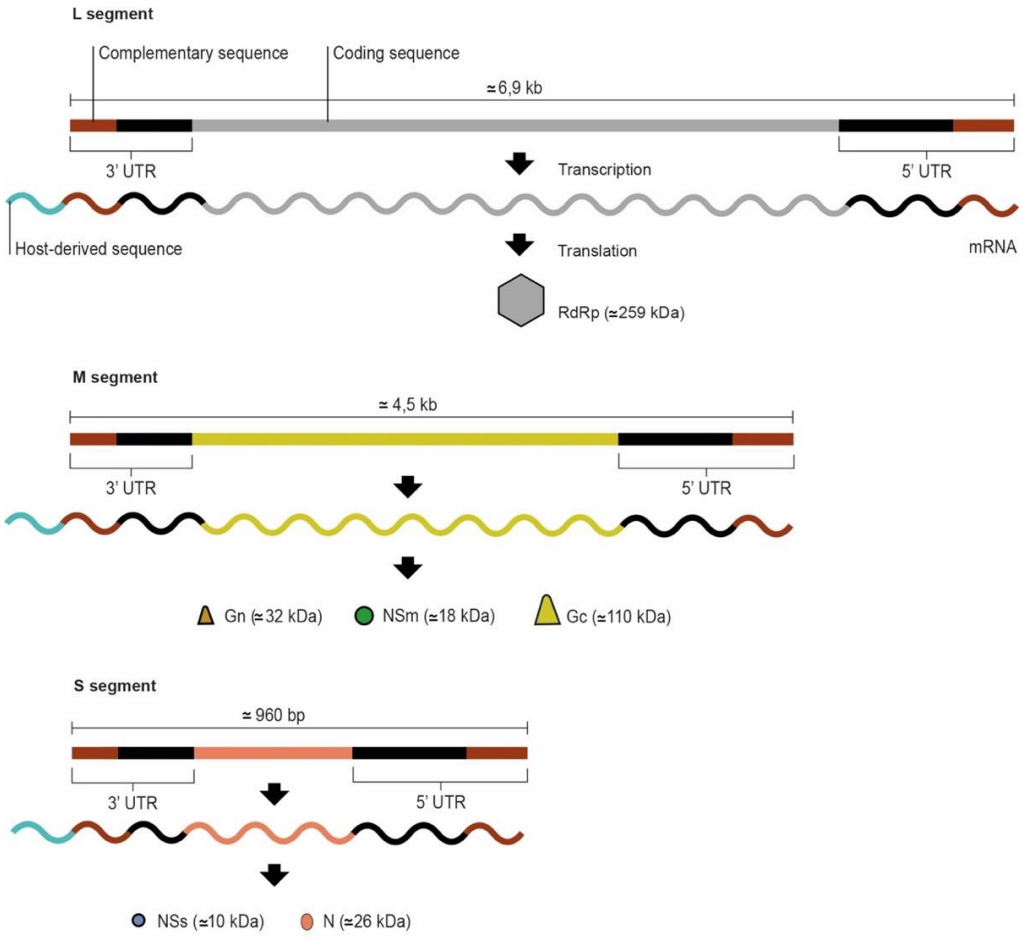
In 2012, Perdões virus was isolated in a municipality with the same name, in Minas Gerais State, Brazil from the liver of a dead black-tufted marmoset (*Callitrhix penicillata*). The virus was also described as a reassortant of OROV (Tilston-Lunel *et al.*, 2015).

Many orthobunyaviruses have yet to be fully sequenced and there are probably many others that have yet to be detected (Ladner *et al.*, 2014).

### 3.1.2. Genome organisation

The genome of orthobunyaviruses consists of three segments of single-stranded, negative-sense RNA, designated large (L), medium (M) and small (S), of approximately 6.9kb, 4.5kb and 1.0kb, respectively. These three genomic segments encode a total of six proteins (Elliott, 1990). All three segments include a coding region flanked by 3´ and 5´ untranslated regions (UTRs). The terminal eleven nucleotides at both ends are conserved and complementary inverted, facilitating the formation of pan-handle structures, which function as the promoter for the transcription and replication of each segment (Kohl, Dunn, Lowen, & Elliott, 2004; Barr & Wertz, 2005; Elliott & Blakqori, 2011; Elliott, 2014) (Figure 3.2).

**Figure 3.2. Genome organisation of *Orthobunyavirus* (Original).**



The L segment encodes the viral RNA-dependent RNA polymerase (RdRp) (Saeed *et al.*, 2001b; Elliott & Blakqori, 2011; Chowdhary *et al.*, 2012), that catalyses both transcription and genome replication. In addition, it contains an endonuclease domain that cleaves capped oligomers from host mRNA, which is used to prime viral mRNA synthesis, a phenomenon known as cap snatching (Reguera, Weber, & Cusack, 2010; Elliott, 2014).

The M segment encodes a precursor polyprotein which is post-translational cleaved by host proteases, giving rise to the surface glycoproteins Gn and Gc, as well as a non-structural protein, NSm (Gentsch & Bishop, 1979; Elliott, 1985; Fazakerley, Gonzalez-Scarano, Strickler, Dietzschold, Karush, & Nathanson, 1988; Gerbaud, Pardigon, Vialat, & Bouloy, 1992; Schmaljohn & Hooper, 2001; Elliott & Blakqori, 2011). Viral glycoproteins encoded on the M segment play a crucial role in viral attachment and membrane-fusion. Both glycoproteins are type I integral membrane proteins and form a heterodimer in the endoplasmic reticulum (ER) of the infected cell (Schmaljohn & Hooper, 2001; Nichol *et al.*, 2005; Shi, Brauburger, & Elliott, 2005; Elliott, 2014; Fischer, 2014). When Gc is expressed alone, it is retained in the ER, which suggests that Gn functions as a chaperone for the correct trafficking of Gc to the Golgi complex before viral budding (Shi, Lappin, & Elliott, 2004). It is suggested that NSm may be involved in the processes of viral assembly and budding (Lappin, Nakitare, Palfreyman, & Elliott, 1994). Of all three genomic segments, the M segment has been shown to be the most variable RNA segment of orthobunyaviruses (Kobayashi, Yanase, Yamakawa, Kato, Yoshida, & Tsuda, 2007; Wernike & Beer, 2017).

On the other hand, the S segment is the most conserved one (Saeed *et al.*, 2000; Nunes *et al.*, 2005; Acrani, Gomes, Proenca-Modena, da Silva, Carminati, Silva, Santos, & Arruda, 2010; Vasconcelos, Nunes, Casseb, Carvalho, Pinto da Silva, Silva, Casseb, & Vasconcelos, 2011; Hang, Forshey, Yang, Solorzano, Kuschner, Halsey, Jarman, & Kochel, 2014; Cardoso, Serra, Heinen, Zuchi, Souza, Naveca, Santos, & Slhessarenko, 2015). It encodes two proteins in alternative overlapping open reading frames (ORFs), the nucleocapsid (N) protein and a non-structural protein (NSs), which are translated from the same mRNA by leaky ribosomal scanning (Elliott, 2014). The N protein, the most abundant in the virion and infected cells, encapsidates one copy of each RNA segment, forming a ribonucleoprotein (RNP) complex, which in turn is associated with the RdRp (Elliott & Blakqori, 2011; Walter & Barr, 2011; Elliott, 2014). The RNPs are packaged in a lipid envelope, derived from the host Golgi complex, which is then modified by insertion of the viral glycoproteins, forming a trimer that create the spikes on the virion (Elliott, 2014). The NSs protein is known to be involved in the regulation of translation, apoptosis, viral polymerase activity as well as antagonism of the interferon (IFN) and shutoff of host protein synthesis in mammalian cells (van Knippenberg, Carlton-Smith, & Elliott, 2010; Elliott, Blakqori, van Knippenberg, Koudriakova, Li, McLees, Shi, & Szemiel, 2013; Barry, Varela, Ratinier, Blomström, Caporale, Seehusen, Hahn, Schnettler, Baumgärtner, Kohl, & Palmarini, 2014; Gouzil, Fablet, Lara, Caignard, Cochet, Kundlacz,

Palmarini, Varela, Breard, Sailleau, Viarouge, Coulpier, Zientara, & Vitour, 2017), but also seems to play a role in the replication mechanism in the vector (Szemiel, Failloux, & Elliott, 2012).

The segmented nature of the genome facilitates the possibility of evolution through genetic reassortment (Bowen, Trappier, Sanchez, Meyer, Goldsmith, Zaki, Dunster, Peters, Ksiazek, Nichol, & Force, 2001; Saeed *et al.*, 2001b; Gerrard, Li, Barrett, & Nichol, 2004; Briese, Bird, Kapoor, Nichol, & Lipkin, 2006; Aguilar *et al.*, 2011). This phenomenon occurs when genetically closely-related viruses infect a susceptible cell at the same time, resulting in a progeny virus containing a mixture of genomic L, M, and S segments from the two parental viruses (Travassos da Rosa *et al.*, 2017). However, despite the hypothetical potential for changes in pathogenicity or host range, the role of segment reassortment is largely unknown due to the lack of comprehensive sequence data (Gerrard *et al.*, 2004).

## 3.2. Molecular diagnosis using real-time polymerase chain reaction (qPCR)

### 3.2.1. General review

In virology, pathogen identification and confirmation of infection relies traditionally on virus isolation in susceptible laboratory animals, cultured cell lines or embryonated eggs. Immunological diagnostic methods are also used widely, based on specific antigen-antibody interaction. Molecular diagnostic methods detect or analyse the genotype characteristics of the microorganism and have been gaining more prominence in research and diagnostic laboratories, complementing, or in some cases substituting conventional methods, which are often more laborious, e.g. culture in cell lines (Cunha & Inácio, 2014).

The polymerase chain reaction (PCR) uses a pair of synthetic oligonucleotides or primers, which hybridise to one strand of the double-stranded DNA (dsDNA) target. This hybridised primer is a substrate for a thermostable DNA polymerase to synthesise the complementary strand through sequential addition of deoxynucleotides (dNTPs). The reaction comprises three distinct steps: denaturation of the dsDNA at 94-97°C; annealing of the primers at 50°-75°C and extension at 72-78°C. The ramp rate, the time of incubation at each temperature and the number of cycles is adjusted in a programmable thermal cycler. The conventional detection and evaluation of the amplified product is dependent on a post-PCR electrophoresis in the presence of a DNA stain and analysis of the resulting band after ultraviolet (UV) illumination (Saiki, Scharf, Faloona, Mullis, Horn, Erlich, & Arnheim, 1985; Mullis & Faloona, 1987; Peake, 1989; Mackay *et al.*, 2002).

Real-time PCR (qPCR) enables simultaneous detection of the target and monitoring of the amplification dynamics, decreases procedure time, eliminates the necessity of post-reaction manipulation and makes possible the quantitative analysis of the product. Observation of the

accumulating amplicon relies on the usage of fluorogenic molecules (Holland, Abramson, Watson, & Gelfand, 1991; Mackay *et al.*, 2002; Tenreiro, Chaves, & Tenreiro, 2014).

One of the variety of chemistries utilised for the detection of fluorescence during the qPCR reaction is the hydrolysis probe, also called a TaqMan® probe or 5' nuclease oligonucleotide probe. This probe is dual-fluorophore labelled with a reporter at the 5' terminus and a quencher at the 3' terminus. When in close proximity, the photon-induced excited 5' reporter fluorophore transfers energy by Förster resonance energy transfer (FRET) to the 3' quencher fluorophore when the probe is intact. However, when the probe is hydrolised due to the 5' exonuclease activity of the TaqMan® DNA polymerase, the reporter energy emission is no longer quenched, resulting in detectable fluorescence (Holland *et al.*, 1991). Hydrolysis probes have strict design requirements, such as: a length of 20-40 nt; a guanine-cytosine content (GC-content) of 40-60%; no single nt repetitions, especially G; a melting temperature ($T_M$) at least 5$^oC$ higher than the primers, in order to ensure that the probe hybridises to the template before the extension (Wittwer, 2001). Recently, an enhancement was made to the hydrolysis probe, the so-called minor groove binder (MGB) probes, that incorporate a 3' nonfluorescent quencher (NFQ), which results in lower background signal and subsequent higher precision in quantitation, but also a MGB moiety that stabilises the probe-template duplex by folding into the minor groove of the dsDNA, thus permitting the use of shorter probes (14 nt), providing better sequence discrimination and flexibility to accommodate more targets (Kutyavin, Afonina, Mills, Gorn, Lukhtanov, Belousov, Singer, Walburger, Lokhov, Gall, Dempcy, Reed, Meyer, & Hedgpeth, 2000).

qPCR has received widespread approval and recognition across a wide range of scientific specialities, due to the high sensitivity of the assay, reduced risk of carry over contamination, improved speed to perform the assay, possibility of type isolates, multiplex capacity and an ability to quantitate precisely the nucleic acids (Mackay *et al.*, 2002; Mosammaparast & McAdam, 2013). It has become one of the most widely used methods for direct virus detection (Wernike & Beer, 2017).

### 3.2.2. Simbu molecular diagnostic assays based on real-time RT-PCR

The isolation, consequent identification and characterisation of "*Bunyavirus"* isolates have been performed historically using mostly antibody based techniques, including HI, CF, SNT, immunofluorescence (IF) tests and more recently enzyme-linked immunosorbent assays (ELISA) (Shope & Causey, 1962; Lanciotti & Tsai, 2007; Mathew, Klevar, Elbers, van der Poel, Kirkland, Godfroid, Mdegela, Mwamengele, & Stokstad, 2015). While these methods are essential for virus identification based on a group-specific approach, they can be time-consuming and restricted in their ability to generate unequivocal and species-identifying results, due to antibody cross-reactivity. Modern molecular technologies and its application in diagnostic reference laboratories provide a time-efficient alternative to traditional serology in

the identification of virus isolates (Lambert & Lanciotti, 2009), without requiring virus isolation or standardised serological reagents (Shaw, Monaghan, Alpar, Anthony, Darpel, Batten, Guercio, Alimena, Vitale, Bankowska, Carpenter, Jones, Oura, King, Elliott, Mellor, & Mertens, 2007).

As the clinical signs caused by some of the *Orthobunyavirus* can be very similar, confirmatory diagnosis and species identification always require viral detection, in order to differentiate infection between the viruses on the serogroup (Lee, Seo, Park, Kim, Cho, Kim, Cho, & Jeoung, 2015).

So far, many TaqMan® based real-time reverse transcription PCR (RT-qPCR) assays have been developed for the detection of arboviruses (Garcia, Crance, Billecocq, Peinnequin, Jouan, Bouloy, & Garin, 2001; Dyer, Chisenhall, & Mores, 2007; Rodriguez-Sanchez, Fernandez-Pinero, Sailleau, Zientara, Belak, Arias, & Sanchez-Vizcaino, 2008; Barros, Ramos, Zé-Zé, Alves, Fagulha, Duarte, Henriques, Luís, & Fevereiro, 2013; Pang, Li, Li, Qu, He, Zhang, Li, Zhang, Liang, & Li, 2014), including assays for agents belonging to the genus *Orthobunyavirus* (Stram, Kuznetzova, Guini, Rogel, Meirom, Chai, Yadin, & Brenner, 2004b; Fischer *et al.*, 2013; Rodrigues Hoffmann, Dorniak, Filant, Dunlap, Bazer, de la Concha-Bermejillo, Welsh, Varner, & Edwards, 2013; Van Eeden, Zaayman, & Venter, 2014b; Lee *et al.*, 2015; Shirafuji *et al.*, 2015).

Stram *et al*. designed a multiplex real-time RT-PCR for the detection and quantitation of AKAV and AINOV using two Taqman® MGB probes, each labelled with different fluorescent dyes for the discrimination of both viruses (Stram *et al.*, 2004b).

Fischer *et al.* developed a pan-Simbu real-time RT-PCR system based on two conserved sequence regions of the L segment, facilitating the amplification of a 279-base pair (bp) product. The assay used a single set of primers and a SYBR® Green I dye (Fischer *et al.*, 2013).

Van Eeden *et al.* described a nested real-time RT-PCR for the detection of SHUV, targeting a conserved region of the S segment, using two pairs of primers and a set of hybridisation probes (Van Eeden *et al.*, 2014b).

Shirafuji *et al.* developed a multiple TaqMan® assay based on conserved regions of the S segment of AKAV, AINOV, PEAV, SATV and SHAV. An universal set of primers and a TaqMan® probe were designed for the broad-range detection of Simbu serogroup lineage 1 (Saeed *et al.*, 2001a) but targeting mainly the five arboviruses mentioned above (Shirafuji *et al.*, 2015).

Lee *et al.* used a common set of specific primers and three different virus-specific probes in order to amplify the genes encoding the nucleocapsid protein (S segment) of AKAV, AINOV and SBV in a one-step multiplex reverse-transcriptase quantitative PCR assay (mRT-qPCR) (Lee *et al.*, 2015).

Recently, Naveca *et al.* described a multiplex real-time RT-PCR able detect OROV, JATV, MDDV, IQTV and Perdões virus using TaqMan® MGB technology (Naveca, Nascimento, Souza, Nunes, Rodrigues, & Vasconcelos, 2017) and Tauscher *et al.* reported three distinct assays for the specific detection of SIMV, SATV and SABOV (Tauscher, Wernike, Fischer, Wegelt, Hoffmann, Teifke, & Beer, 2017).

After the emergence of SBV in Europe in 2011, many diagnostic laboratories and research institutes all over the continent developed real-time RT-PCR based assays for its detection in a variety of field samples from invertebrate and vertebrate hosts (Bilk, Schulze, Fischer, Beer, Hlinak, & Hoffmann, 2012; De Regge, Deblauwe, De Deken, Vantieghem, Madder, Geysen, Smeets, Losson, van den Berg, & Cay, 2012; Fischer *et al.*, 2013; Aebischer, Wernike, Hoffmann, & Beer, 2014; De Regge, Madder, Deblauwe, Losson, Fassotte, Demeulemeester, Smeets, Tomme, & Cay, 2014; Ponsart, Pozzi, Breard, Catinot, Viard, Sailleau, Viarouge, Gouzil, Beer, Zientara, & Vitour, 2014; Rasmussen *et al.*, 2014; Schulz, van der Poel, Ponsart, Cay, Steinbach, Zientara, Beer, & Hoffmann, 2015; Segard, Gardes, Jacquier, Grillet, Mathieu, Rakotoarivony, Setier-Rio, Chavernac, Cetre-Sossah, Balenghien, & Garros, 2017; Wernike, Beer, & Hoffmann, 2017b).

# 4. Aims and Objectives

## 4.1. Aim

Develop a group-specific RT-qPCR for the detection of the Simbu serogroup orthobunyaviruses.

## 4.2. Objectives

Evaluate the nucleotide variation within the genome of all the Simbu serogroup orthobunyaviruses in published data.

Design specific sequencing primers to perform AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV sequencing and compare the results with other published sequences.

Design and optimise specific primers and probes for the group-specific RT-qPCR for the detection of Simbu serogroup orthobunyaviruses.

Perform laboratory validation of RT-qPCR assay in terms of efficiency, sensitivity, specificity and repeatability.

Test field samples with the developed and optimised assay.

Generate a geographic information system based map of the known distribution of the Simbu serogroup orthobunyaviruses.

# 5. Materials and Methods

## 5.1. Simbu serogroup viral isolates

The Simbu serogroup orthobunyaviruses stored at the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Sciences, University of Pretoria were used for the development and optimisation of the assay (Table 5.1).

All the isolates were identified originally by serology using CF and SNT (Costa Mendes, 1984). The viruses were also tested and confirmed positive using a pan-Simbu real-time RT-PCR (Fischer *et al.*, 2013), with the exception of 8912 #1 BHK – 2/2/96 and Cu 1/70 #1 Vero – 21/7/94.

**Table 5.1. Simbu serogroup orthobunyavirus isolates stored in the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Sciences, University of Pretoria.**

| Name | Virus | Strain | $TCID^{j}_{50}$/ml |
|---|---|---|---|
| Akabane #2 Vero #3 BHK – 22/5/98[a] | AKAV | Ja Gar 39 | $1\times10^{4.9}$ |
| Ar 53 #2 BHK – 2/2/96[b] | SIMV | SA Ar 53 | $1\times10^{5.7}$ |
| Shuni #2 BHK #2 Vero – 22/5/98[c] | SHUV | An 10107 | $1\times10^{4.5}$ |
| Sathuperi #2 BHK – 2/2/96[d] | SATV | IG 10310 | $1\times10^{5.3}$ |
| Shamonda #1 Vero – 21/1/94[e] | SHAV | An 5550 | $1\times10^{5.3}$ |
| Ingwavuma #2 BHK – 2/2/96[f] | INGV | SA An 4165 | $1\times10^{6.5}$ |
| Sabo #2 Vero #1 BHK – 3/8/94[g] | SABOV | AN 9398 | $1\times10^{4.3}$ |
| 8912 #1 BHK – 2/2/96[h] | SHUV | 8912 | - |
| Cu 1/70 #1 Vero – 21/7/94[i] | SABOV | Cu 1/70 | - |

[a] **Isolated in Japan from pools of *Aedes vexans* and *Culex tritaeniorhynchus* mosquitoes in 1959, with to two passages in African green monkey kidney (Vero) cells and three passages in baby hamster kidney (BHK) cells.**
[b] **Isolated in South Africa from *Aedes circumluteolus* mosquitoes caught during 1955 and 1957, with two passages in BHK cells.**
[c] **Isolated in Nigeria from a bovine in 1966, with two passages in BHK cells and two passages in Vero cells.**
[d] **Isolated in India from pools of *Culex vishnui* mosquitoes in 1957, with two passages in BHK cells.**
[e] **Isolated in Nigeria from a bovine in 1965, with one passage in Vero cells.**
[f] **Isolated in South Africa from a spectacled weaver in 1959, with two passages in BHK cells.**
[g] **Isolated in Nigeria from a goat in 1966, with two passages in Vero cells and one passage in BHK cells.**
[h] **Isolated in South Africa from cattle during an outbreak of a formerly unrecognised disease in 1967, with one passage in BHK cells.**
[i] **Isolated in South Africa from *Culicoides* spp. in 1970, with one passage in Vero cells.**
[j] **TCID – tissue culture infectious dose**

### 5.1.1. Infectivity

African green monkey kidney (Vero) cells (ATCC®) were grown in minimum essential medium (MEM) with Earle's balanced salts, L-glutamine and non-essential amino acids (Biowest), supplemented with 2.95% tryptose phosphate broth (TPB) (BD Biosciences), 5% γ-irradiated foetal bovine serum (FBS) (Biowest) and 50 mg/ml gentamicin (Virbac) at 37ºC in a humidified 5% $CO_2$ atmosphere.

The Vero cell suspension was counted in a Neubauer chamber, adjusted to the optimal concentration of $48\times10^4$ cells/ml and plated (80 µl) in flat-bottomed 96-well clear polystyrene plates (ThermoFisher).

A $\log_{10}$ dilution series was carried out for Akabane #2 Vero #3 BHK – 22/5/98, Ar 53 #2 BHK – 2/2/96, Shuni #2 BHK #2 Vero – 22/5/98, Sathuperi #2 BHK – 2/2/96, Shamonda #1 Vero – 21/1/94, Ingwavuma #2 BHK – 2/2/96 and Sabo #2 Vero #1 BHK – 3/8/94 viruses in MEM with 5% FBS and gentamicin.

Tenfold dilutions of the viruses mentioned above ($10^{-1}$ up to $10^{-8}$) were inoculated (100 µl) in the plates (five replicates per dilution) and the expected cytophatic effect (CPE) was read five and six days after inoculation using an inverted optical microscope.

The 50% tissue culture infectious dose ($TCID_{50}$) was calculated using the Spearman-Karber method (Spearman, 1908; Kärber, 1931).

## 5.2. Virus distribution

A comprehensive review of a hundred and five published references, including research articles, short communications, scientific reports and book chapters regarding the worldwide distribution of the Simbu serogroup orthobunyaviruses, as well as related seroconversion, was carried out using Google search, Google Scholar and National Centre for Biotechnology Information's (NCBI) PubMed. The search terms were the names of each of the thirty-two viruses of the Simbu serogroup. Abstracts were screened for relevance and appropriate papers then read in full. The primary papers led to other references by way of publications cited in their text.

This data were used as input in order to generate a map of the known distribution of these viruses and reported seroprevalence, using ArcGIS version 10.5 (ESRI, 2016).

## 5.3. Bioinformatics

### 5.3.1. Evaluation of genome variation

Nucleotide sequences from the three different segments of the Simbu serogroup orthobunyaviruses available on the NCBI's GenBank® were downloaded and aligned using MAFFT version 7.3.1.3 (Katoh, Rozewicki, & Yamada, 2017). BioEdit Sequence Alignment Editor version 7.2.3 (Hall, 1999) was used to edit the sequences and to visualise conserved regions present in the ORF. The nucleotide sequences were then translated into amino acids and aligned for a second time to ensure a better alignment quality. EMBOSS: tranalign (Rice, Longden, & Bleasby, 2000) was used to back translate the nucleic acid sequence from the aligned protein. DAMBE software package version 5.3.48 (Xia, 2013) was used to identify identical sequences.

To summarise the results and simplify the observation of the nucleotide variation among the three segments, a variation score was calculated using the equation below and Microsoft® Excel version 15.40 was used to create a plot:

$$Variation = 1 - x_1 + x_2 + 2x_3 + 3x_4$$

$x_i$ – Frequency of the most common nucleotides; (1≤ i ≤4)

### 5.3.2. Primer and probe design

#### *5.3.2.1. Sequencing primers*

Seven primer sets were designed using the PrimerQuest® tool (Integrated DNA Technologies, Inc.) for the full-length sequencing of the S segment of AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV (sequences available at GenBank®).

The primers were synthesised by Integrated DNA Technologies, Inc., delivered dried, re-suspended in Tris-EDTA buffer (TE buffer) to a 100 μM stock concentration and working aliquots at 20 μM were produced by dilution with ultrapure 18.2 MΩ.cm 25°C water (Elix® Essential 5 and Synergy® water purification systems, Merck).

#### *5.3.2.2. Real-time RT-PCR primers and probes*

After determining the nucleotide variation within the genome, a conserved region situated between the nucleotide positions 120 - 242 of the S segment was elected to design a serogroup-specific universal primer set, Simbu_F and Simbu_R, targeting all the Simbu orthobunyaviruses with available sequence data. Due to the nucleotide distinctiveness between phylogenetic clades, two different TaqMan® MGB probes (Applied Biosystems®) were designed, one targeting clade A viruses, Simbu_CladeAP, labeled with a VIC® fluorescent dye, and another targeting clade B viruses, Simbu_CladeBP, labeled with a FAM™ (6-carboxyfluorescein) fluorescent dye. Primer Express® version 2.0 (Applied Biosystems®) was used to ensure optimal values in terms of GC-content, $T_M$ and to avoid the formation of secondary structures. *In silico* specificity screening was carried out using NCBI's Basic Local Alignment Search Tool (BLAST®) to prevent non-specific reactions.

The primer set and MGB probes were synthesised by Integrated DNA Technologies, Inc. and ThermoFisher Scientific, Inc., respectively. The TE buffer-re-suspended primers and the 100 μM stock probe were both aliquoted to a 20 μM working concentration, by dilution with ultrapure 18.2 MΩ.cm 25°C water (Elix® Essential 5 and Synergy® water purification systems, Merck).

## 5.4. Nucleic acid purification

The viral RNA from the DVTD's isolates was extracted with a magnetic bead based separation method using the KingFisher™ Duo Prime Purification System (ThermoFisher) with the

MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems®), according with the manufacturer's instructions. A standard protocol (MagMAX_Pathogen_Stnd_Vol_DUO.bdz) was used. The nucleic acid was eluted in 50 µl Elution Buffer.

## 5.5. Sequencing

The S segments of the isolates stored in DVTD were sequenced for the first time.

A conventional PCR was conducted utilising the SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase protocol (Invitrogen™), which consisted of a 50 µl total reaction volume composed of 25 µl 2X Master Mix, 2 µl SuperScript® III RT/Platinum® *Taq* Mix, 0.5 µl of each 20 µM primer, 2 µl RNA template and nuclease-free water to the final volume. The reactions were performed in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems®) and the reaction conditions were 55ºC for 30 min to reverse transcribe the RNA, 94ºC for 2 min for reverse transcriptase (RT) inactivation, followed by 40 cycles of 94ºC for 15 s (dsDNA denaturation), 55ºC for 30 s (primer annealing) and 68ºC for 1 min (extension).

In order to confirm the correct fragment size, gel electrophoresis was carried out with a 2% agarose gel, using 1×Tris-acetate-EDTA (TAE) buffer in the presence of ethidium bromide stain. 5 µl GeneRuler 100 bp DNA Ladder, ready-to-use (ThermoFisher) was added to the first well, 1 µl 6× DNA Loading Dye (ThermoFisher) per 5 µl of sample was added to the remaining wells. The gel was electrophoresed at 100 V. To visualise and record the obtained bands, a ChemiDoc™ XRS+ System with Image Lab™ Software version 3.0 (Bio-Rad) was used.

The PCR products were purified using the CleanSweep™ PCR purification reagent (Applied Biosystems®) in order to dephosphorylate unincorporated nucleotides and digest unused primers, ensuring accurate downstream sequencing data. The purification protocol was accomplished in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems®) and the conditions were 37ºC for 15 min, followed by 80ºC for 15 min.

Sequencing was performed by Inqaba Biotec™ (Pretoria, RSA), using the Sanger method (Sanger, Nicklen, & Coulson, 1977) with the same primers used for PCR product amplification.

The resultant sequencing data was then analysed, edited and assembled using Pregap4 and Gap4 of the Staden Software Package version 1.5 (Staden, F. Beal, & K. Bonfield, 2000).

## 5.6. The group-specific RT-qPCR assay

In order to allow the rapid, broad, sensitive and specific detection of the Simbu serogroup orthobunyaviruses, a group-specific TaqMan® based RT-qPCR assay was developed, optimised and validated.

All the reactions were set up manually in either MicroAmp™ Optical 8-Cap Strips (ThermoFisher) or MicroAmp™ Fast Optical 96-Well Reaction Plates, 0.1 mL (ThermoFisher) and performed using the TaqMan® Fast Virus 1-Step Master Mix (ThermoFisher) protocol in a StepOnePlus™ Real-Time PCR System (Applied Biosystems®). All the generated data was analysed using the StepOne™ Software version 2.3 (Applied Biosystems®). Reactions were conducted in a 20 μl total volume of which 5 μl were 4×TaqMan® Fast Virus 1-Step Master Mix (MM) and 2 μl of template RNA. Primer, probe and nuclease-free water volumes varied according to the different primer and probe concentrations used for the development and optimisation of the assay and will be specified later. The thermal-cycling conditions comprised a holding stage of 50ºC for 5 min for reverse transcription, 95ºC for 20 s for RT inactivation and initial dsDNA denaturation, followed by 40 cycles of 95ºC for 3 s for denaturation and 60ºC for 30 s for primer annealing and extension.

## 5.6.1. Development

### *5.6.1.1. Pilot run*

A pilot experiment was carried out in order to confirm primer and probe capability and resultant amplification. The RNA from DVTD's isolates AKAV, SIMV, SHUV, SATV, SHAV, SABOV viruses was used as target for probe Simbu_CladeBP detection and the RNA from INGV as a target for probe Simbu_CladeAP detection. The reaction contained 4×MM, 2 μl RNA, 900 nM of each primer, 250 nM of each probe and nuclease-free water to a final reaction volume of 20 μl.

### *5.6.1.2. Comparison with a pan-Simbu assay (Fischer et al., 2013)*

This group-specific real-time RT-PCR was compared with a pan-Simbu real-time RT-PCR (Fischer *et al.*, 2013).

Fischer's assay was conducted with a reverse transcription step using the Omniscript® Reverse Transcription Kit (QIAGEN) in a 20 μl reaction volume, which comprised 2 μl 10× Buffer RT, 2 μl dNTPs mix, 2 μl 10 μM random primers, 1 μl Omniscript® RT, 2 μl template RNA and nuclease-free water. The reaction was run in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems®) for 60 min at 37ºC. The subsequent step involved a SYBR® Green I based real-time PCR with the KAPA SYBR FAST qPCR Master Mix (2×) ABI Prism™ (Kapa Biosystems) as follows: 10 μl 2× Master Mix, 1 μl 10 μM of each primer (panOBV-L-2959 F and panOBV-L-3274R), 2 μl cDNA and nuclease-free water to a final volume of 20 μl. The real-time reaction was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems®) and the thermal cycling conditions consisted of a holding stage of 95ºC for 20 s followed by 40 cycles of 95ºC for 3 s, 55ºC for 20 s and 72ºC for 10 s. The melting curve was

acquired with the following steps: 95ºC for 15 s, 55ºC for 1 min, data collection with increase of temperature at 0.3%, and 95ºC for 15 s.

The comparison was conducted using a $10^{-1}$ dilution of AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV RNA for each experiment.

## 5.6.2. Optimisation

### *5.6.2.1. Primer concentration*

In order to optimise the forward and reverse primer concentration in the RT-qPCR reaction, four different primer concentrations were tested with a constant probe concentration of 250 nM: 100 nM, 200 nM, 400 nM and 800 nM.

### *5.6.2.2. Simbu_CladeAP probe concentration*

To optimise the Simbu_CladeAP probe concentration in the reaction, seven different probe concentrations were tested with a constant primer concentration of 200 nM: 50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 500 nM and 750 nM.

In addition, a supplementary run was performed using three different primer concentrations, 200 nM, 400 nM and 800 nM against two different probe concentrations, 250 nM and 500 nM.

### *5.6.2.3. Simbu_CladeBP probe concentration*

To optimise the Simbu_CladeBP probe concentration in the reaction, five different probe concentrations were tested with a constant primer concentration of 200 nM: 50 nM, 100 nM, 150 nM, 200 nM and 250 nM.

## 5.6.3. Efficiency

The PCR efficiency was determined for the cell cultured AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV. A ten-fold eight-log serial dilution was made for the seven viruses in nuclease-free water and each dilution was tested in triplicate. Concentrations of primers, probe Simbu_CladeAP and probe Simbu_CladeBP in the reactions were 400 nM, 500 nM and 250 nM, respectively. The amplification efficiency was then calculated from the slope of the linear regression between log $TCID_{50}$/reaction and $C_q$ values using the following formula:

$$Efficiency\ (\%) = \left(10^{\left(\frac{-1}{Slope}\right)} - 1\right) \times 100$$

## 5.6.4. Sensitivity

Subsequent to the $\log_{10}$ serial dilution made for efficiency determination, a two-fold dilution series was carried out with $10^{-5}$ AKAV, $10^{-5}$ SIMV, $10^{-5}$ SHUV, $10^{-5}$ SATV, $10^{-3}$ SHAV, $10^{-3}$ INGV

and $10^{-3}$ SABOV in nuclease-free water. The dilution series was tested four times in a single run and the results utilised to calculate the 95% limit of detection (LOD) of the assay by probit analysis, using SPSS® Statistics version 25 (IBM®). From this computer-based regression modelling, the analytical sensitivity, which is defined as the viral titre detected 95% of the time, was calculated.

### 5.6.5. Specificity

Nucleic acid of genetically related, causative agents of abortion in ruminants and arthropod-borne viruses such as *Pestivirus A* (BVDV-1, V27/04 C #2 MDBK 6.12.04), *Bovine alphaherpesvirus 1* (BoHV-1, #3 MDBK 12.10.00, IBR American strain), *Bluetongue virus* (BTV-1, pp 28.1.98), *Bovine fever ephemerovirus* (BEFV, #2 BHK 20.9.95, Van der Westhuizen strain), *Rift Valley fever phlebovirus* (RVFV, TC50+ #2 Vero 31.5.95), *Wesselsbron virus* (WSLV, #9 Vero 8.6.15) and *Palyam virus* (PALV, Kasba #1 Vero 19.2.16) was used to determine the assay specificity. Viral nucleic acid was extracted using the KingFisher™ Duo Prime Purification System (ThermoFisher) with the MagMAX™ Pathogen RNA/DNA Kit (ThermoFisher) according with the manufacturer's instructions and was used as a template along with a clade A and clade B virus control. *In silico* specificity was also performed by BLAST® analysis.

### 5.6.6. Repeatability

The repeatability of the assay was determined by repeating the experiment used for the sensitivity determination (5.6.4) four times, in separate runs. INGV and SATV were used as templates to represent Simbu clades A and B, respectively. The data generated were used to assess the intra-run, inter-run and overall variation for both probe Simbu_CladeAP and Simbu_CladeBP detection.

### 5.6.7. Testing of field samples

#### *5.6.7.1. Abortion products from Namibia*

In 2017, around Windhoek, Republic of Namibia, an abortion storm affected all species of domestic ruminants, with more than a hundred abortions reported in cattle. Anatomopathological examination revealed meconium-stained foetuses with pleural and peritoneal sanguinolent effusion, abdominal organs in an advanced stage of autolysis, associated with necrotic placentitis. Further investigation of the aetiology included laboratorial diagnosis for common infectious causes of abortion in ruminants, such as *Brucella* spp., *Leptospira interrogans, Coxiella burnetii, Listeria monocytogenes, Chlamydia* spp., *Campylobacter fetus, Tritrichomonas foetus*, BoHV-1, BVDV-1, RVFV and all tested negative.

Samples of pooled organs and brain from five different bovine foetuses, namely 5709, 5737, 5738, 5697 and 5030 were tested for Simbu serogroup orthobunyaviruses with the group-specific real-time RT-PCR. Scalpel blade cut sections of pooled organs and brain (approximately 20 mg) were placed in MagNA Lyser Green Beads tubes (Roche Life Science) containing 500 µl phosphate buffered saline (PBS), homogenised in a Precellys®24 (Bertin Corp) tissue homogeniser at 6700 rpm for 2×30 s and then centrifuged at 1000 rpm for 1 min in a 5417R centrifuge (Eppendorf®). The nucleic acid in the supernatant was purified using the simple workflow extraction protocol, the KingFisher™ Duo Prime Purification System (ThermoFisher) and the MagMAX™ CORE Nucleic Acid Purification Kit (Applied Biosystems®), according with the manufacturer's instructions. The nucleic acid extracted from the pooled organs and brain was tested separately. The real-time RT-PCR protocol comprised 4× master mix, 400 nM of each primer, 500 nM probe Simbu_CladeAP, 250 nM probeSimbu_CladeBP, 2 µl template RNA and nuclease-free water to make up a reaction volume of 20 µl. A protocol for bovine β-actin using custom designed primers and probe was run in parallel as an internal control in a 20 µl reaction as follows: 4×MM, 900 nM of each primer, 250 nM probe, 2 µl of template and nuclease-free water to make up a final volume of 20 µl. Mastermix with no template RNA (negative control) and AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV isolates (positive controls) were included to monitor the occurrence of false positive or false negative results.

### *5.6.7.2. Calf with neurological signs*

In January 2018, in Komatipoort, South Africa, several young calves and lambs exhibited similar clinical signs and macroscopic anatomopathological findings. Clinical examination showed neurological symptomatology characterised by head pressing, fasciculations and ataxia while *post mortem* examination showed pleural and peritoneal effusion, pulmonary oedema, cerebral oedema associated with passive congestion as well as severe kidney lesions.

Samples from a calf, specifically pooled organs, blood and brain were submitted to the DVTD and were tested with the group-specific real-time RT-PCR for Simbu serogroup orthobunyaviruses. Nucleic acid was purified and PCR performed from pooled organs, blood, cortex and cerebellum fragments, as described in 5.6.7.1.

Mastermix with no template RNA was used as negative control. AKAV and INGV RNA were used as clade B and clade A positive controls, respectively.

### *5.6.7.3. Mosquitoes from Bumbe lake*

Thirty pools of fifty morphologically-identified female *Culex pipiens* mosquitoes collected in the Bumbe lake pan, Kwa-Zulu Natal, South Africa, using an Onderstepoort Veterinary Institute

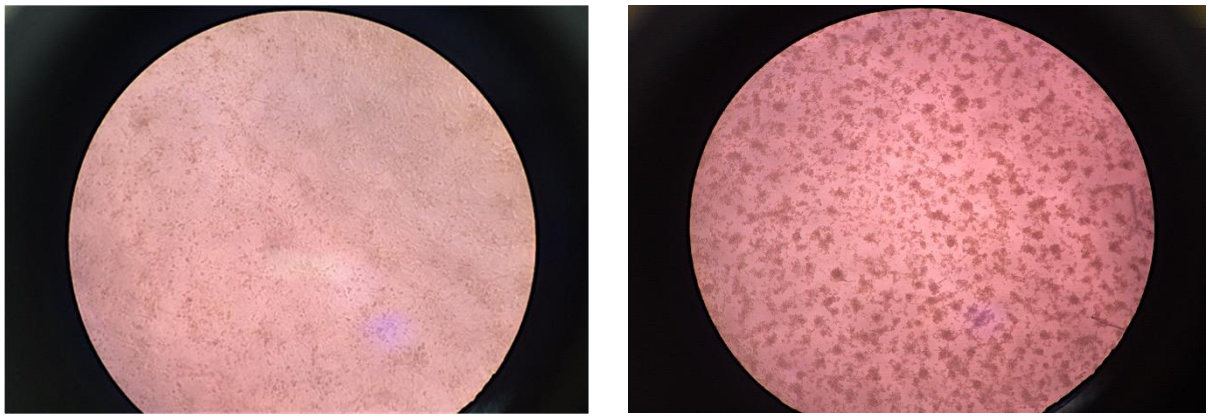
(OVI) trap, were subjected to a nucleic acid purification protocol, as described in 5.6.7.1. To test for clade B Simbu serogroup orthobunyaviruses, the pools were tested with the group-specific real-time RT-PCR with the following protocol: 4× master mix, 400 nM of each primer, 250 nM of probe Simbu_CladeBP, 2 μl of template and nuclease-free water to a final volume of 20 μl. Mastermix with no template RNA and SIMV RNA were used as negative and positive controls, respectively.

# 6. Results and Discussion

## 6.1. Infectivity

The $TCID_{50}$ in Vero cell cultures was determined for each isolate, six days after viral inoculation. CPE caused by Simbu serogroup viruses was identified as cytoplasmic shrinking and cell detachment (Figure 6.1). The obtained log $TCID_{50}$/ml values ranged from 4.3 to 6.5 (Table 6.1).

**Figure 6.1. Effect of Simbu viral infection in Vero cells (400×).**



**Vero cells cultured on 96-well plate under inverted optical microscope (400×). The left well represents the negative control (w/o virus inoculation), whereas the right well respresents the cytophatic effect (CPE) caused by the virus six days after inoculation. The CPE is charaterised by cell shrinking and detachment. The medium color may change due to the pH alteration (Originals).**

**Table 6.1. Log $TCID_{50}$/ml values**

| Virus | Log $TCID_{50}$/ml |
|---|---|
| AKAV | 4.9 |
| SIMV | 5.7 |
| SHUV | 4.5 |
| SATV | 5.3 |
| SHAV | 5.3 |
| INGV | 6.5 |
| SABOV | 4.3 |

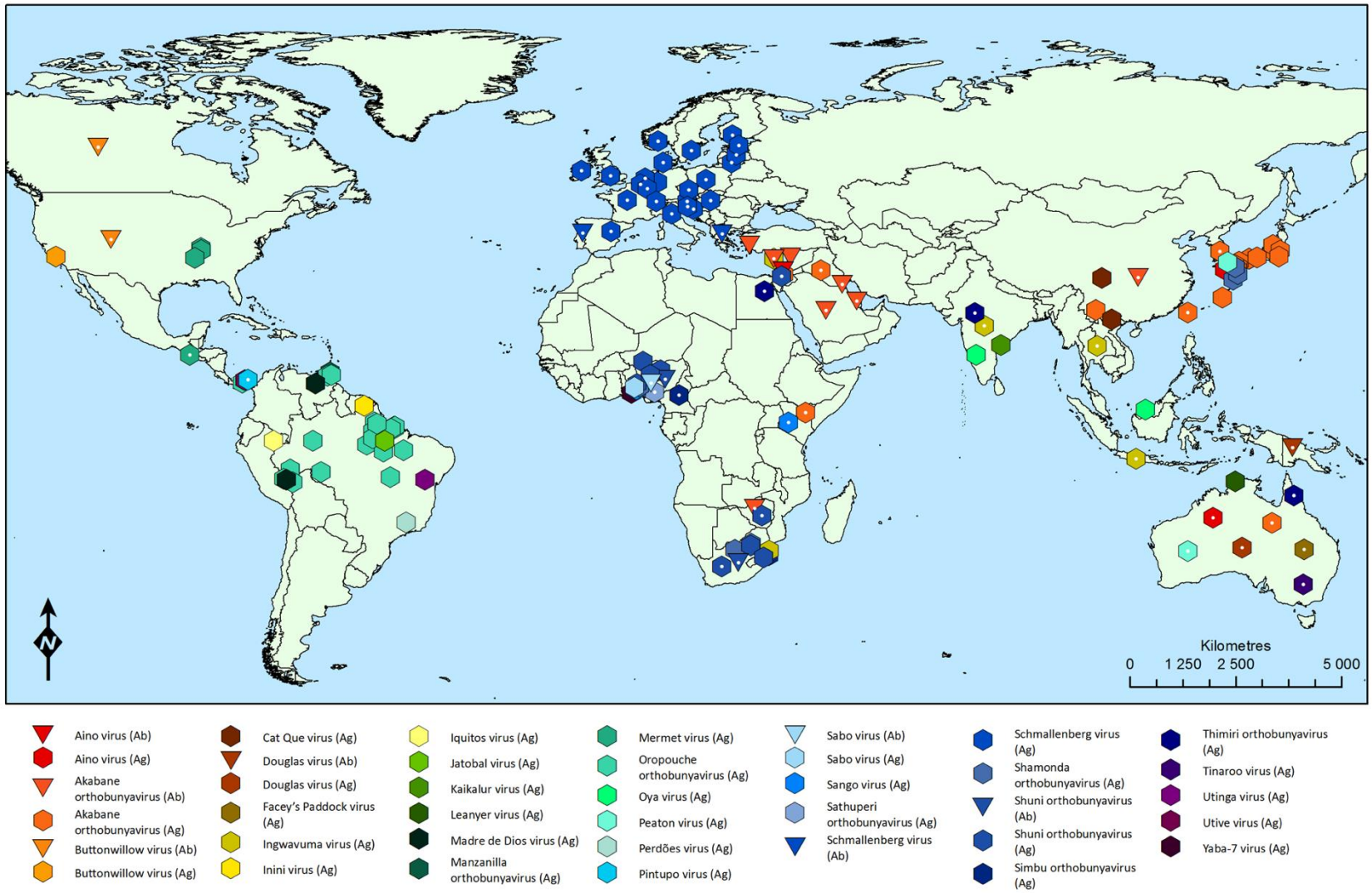
**TCID – tissue culture infectious dose**

## 6.2. Virus Distribution

A geographic information system (GIS) is a framework for gathering, managing, and analysing data. It scrutinises spatial location and organises layers of information into visualisations using maps or tridimensional scenes, helping to generate deeper insights into data, such as patterns, relationships, and trends (ESRI, 2018).

The generated GIS based map (Figure 6.2) shows the known locations of virus isolation and seroconversion against Simbu viruses and makes clear the cosmopolitan, mainly tropical, distribution of the serogroup members. Virus isolation from migratory birds (McIntosh *et al.*, 1965; Calisher *et al.*, 1969; Pavri, 1969; Pajot, 1980; Darwish & Hoogstraal, 1981) and winged

**Figure 6.2. Map of the known distribution of the Simbu serogroup orthobunyaviruses**




**Hexagons represent virus (Ag) isolation and triangles represent antibody (Ab) detection. The white dots indicate that figures were randomly placed in the country whereas absence of white dot indicates the exact location of viral or antibody isolation (Original).**

athropods (Nelson & Scrivani, 1972; Lee, 1979; St George *et al.*, 1980; Standfast & Dyce, 1982; Stram *et al.*, 2004a; De Regge *et al.*, 2012; De Regge *et al.*, 2014) associated with long-distance dispersal (Sanders, 2011; Yanase, 2011; Eagles, Deveson, Walker, Zalucki, & Durr, 2012) might explain the widespread geographical distribution of these viruses. A geographic barrier between viruses of both phylogenetic clades is evident, and even when considered independently, the two Simbu serogroup clades demonstrate substantial geographic distribution. An effort in terms of virus surveillance and identification of arthropod vectors is necessary in order to understand better the factors shaping and maintaining these distinct distributions (Ladner *et al.*, 2014).

## 6.3. Bioinformatics

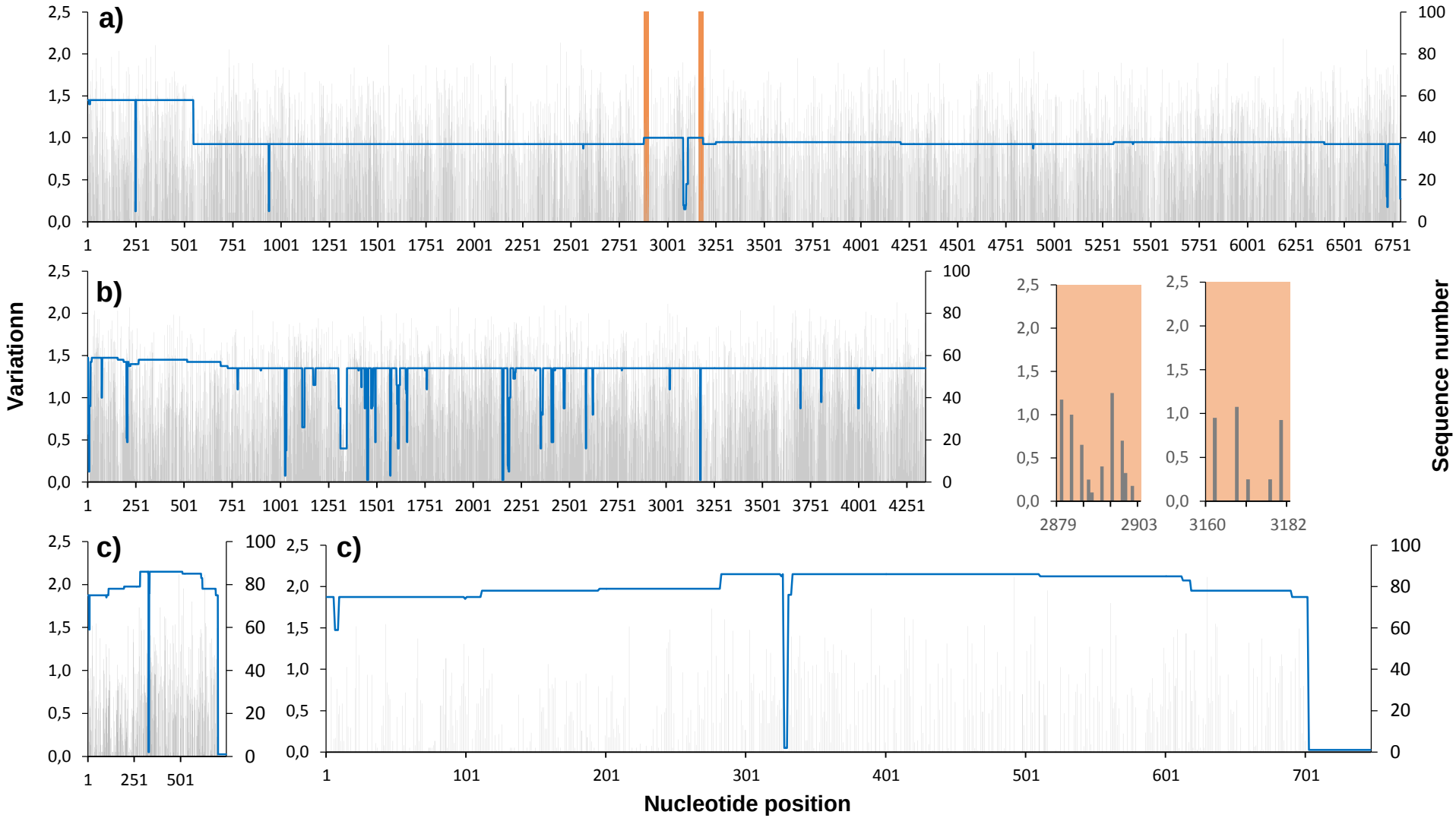
Bioinformatics or computational biology is the direct application of mathematical and computer science methods in order to solve problems in the areas of molecular biology that require the analysis of a substantial number of data (Bry & Kröger, 2003).

### 6.3.1. Evaluation of the genome variation

The assessment of the Simbu serogroup members available genomic data showed a high nucleotide variation along the ORF nucleotide sequences of the three segments, S, M and L (Figure 6.3). Nevertheless, a comparative analysis showed a lower variation score for the S segment, whereas the M segment possessed the highest degree of variation, which corroborates the published literature (Saeed *et al.*, 2000; Nunes *et al.*, 2005; Kobayashi *et al.*, 2007; Acrani *et al.*, 2010; Vasconcelos *et al.*, 2011; Yanase *et al.*, 2012; Hang *et al.*, 2014; Cardoso *et al.*, 2015). The genomic variation between the two distinct phylogenetic clades was also clear, as evidenced by Ladner *et al.* 2014 (see also 6.3.2.2).

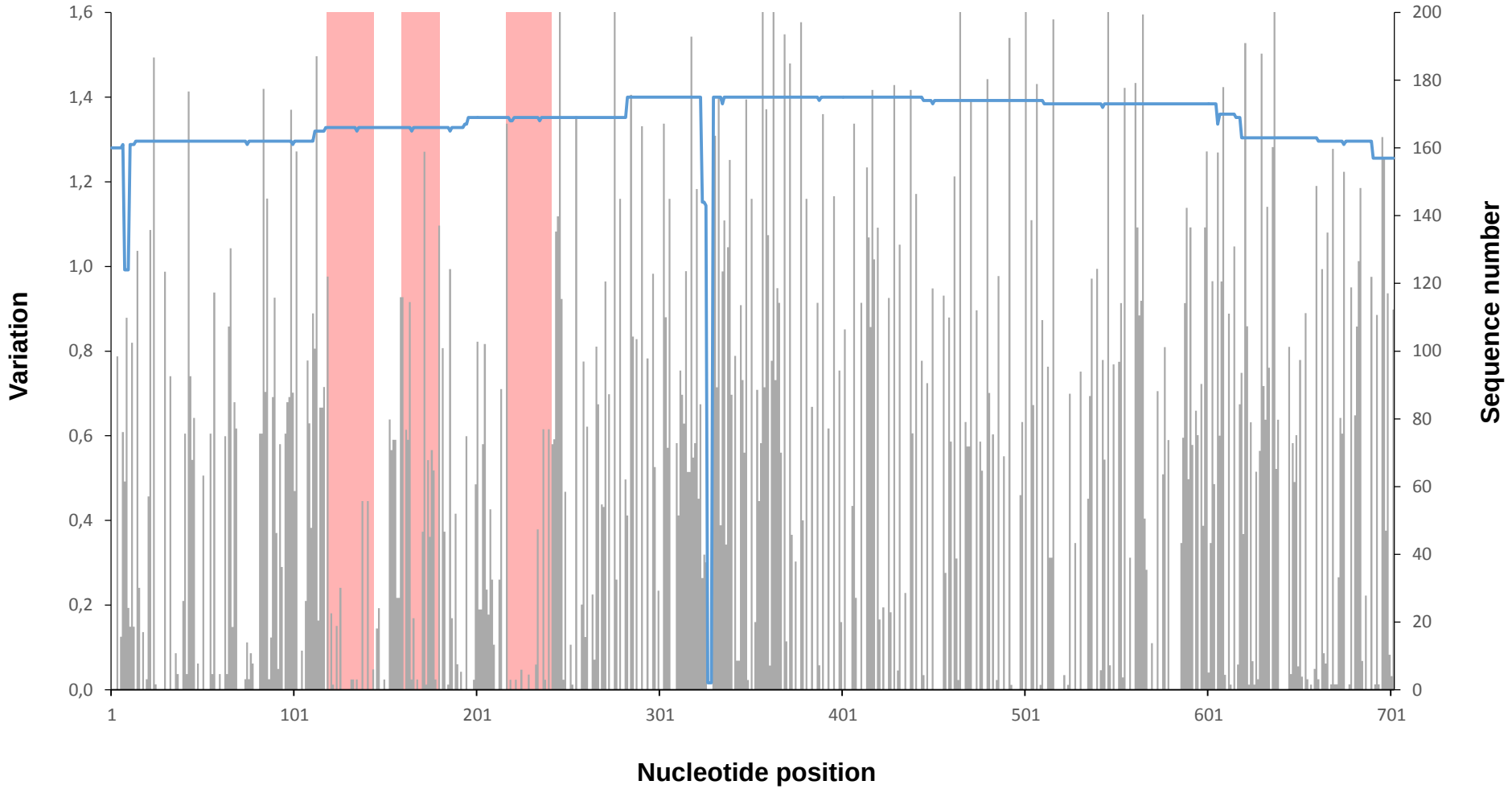
Like all negative-sense RNA viruses, the proofreading function of the *Orthobunyavirus* RdRp is absent, which generates significant genetic heterogeneity in virus populations (Elliott, 2014). In addition, this genetic diversity is suggested to be reasoned on the cosmopolitan distribution of the Simbu serogroup, as other *Orthobunyavirus* serogroups exhibit less genetic diversity, but a narrow geographical range (Saeed *et al.*, 2001a). The diversity between clades is expected, considering that 77% of clade A viruses occur in the New World and no clade B virus have been isolated in this region, as vector and host ranges influence the geographical spread of the microorganisms (Ladner *et al.*, 2014). The high degree of genetic diversity within the segments, particularly S and M, may be because the segments encodes for the N protein and surface glycoproteins (the major immunogens), respectively (Roman-Sosa, Brocchi, Schirrmeier, Wernike, Schelp, & Beer, 2016; Wernike, Aebischer, Roman-Sosa, & Beer, 2017a).

**Figure 6.3. Plot of nucleotide variation in the genome of the Simbu serogroup viruses**




**Plot of nucleotide variation in the L (a), M (b) and S (c) segments of the Simbu serogroup orthobunyaviruses. The S segment is shown twice, first in a view relative to the other segments, and then in an expanded view to better illustrate conserved regions. Column height indicates the variation in each nucleotide position: the higher the column, the greater is the variation. All relevant sequences available in Genbank® were used, and the number of sequences analysed at each nucleotide position is indicated by the blue line. The orange columns indicate the primers used in the pan-Simbu real time RT-PCR assay (Fischer *et al.*, 2013).**

**Figure 6.4. Plot of nucleotide variation in the S segment showing primer and probe position**




**Plot of nucleotide variation within the S segment. Column height indicates the variation in each nucleotide position: the higher the column, the greater is the variation. All relevant sequences available in Genbank® were used, and the number of sequences analysed at each nucleotide position is indicated by the blue line. Red columns indicate the regions targeted by the primers and probe of a Simbu orthbunyavirus group-specific real-time RT-PCR assay, located between the nucleotides 120 - 242.**

It is possible that during the evolution of these viruses, the need to infect and alternate between mammal, bird or insect, may have played a role in the high diversity of the M segment (Saeed *et al.*, 2001a; Yanase *et al.*, 2012). Another explanation is that ancestral viruses may have undergone genetic reassortment (Bowen *et al.*, 2001; Gerrard *et al.*, 2004; Briese *et al.*, 2006; Aguilar *et al.*, 2011), resulting in progeny viruses with similar S segments, but diverse M segments (Saeed *et al.*, 2001a), as it is likely that the N protein and viral polymerase, which are essential for genome replication, to co-evolve together (Elliott, 2014).

## 6.3.2. Primer and Probe Design

### *6.3.2.1. Sequencing primers*

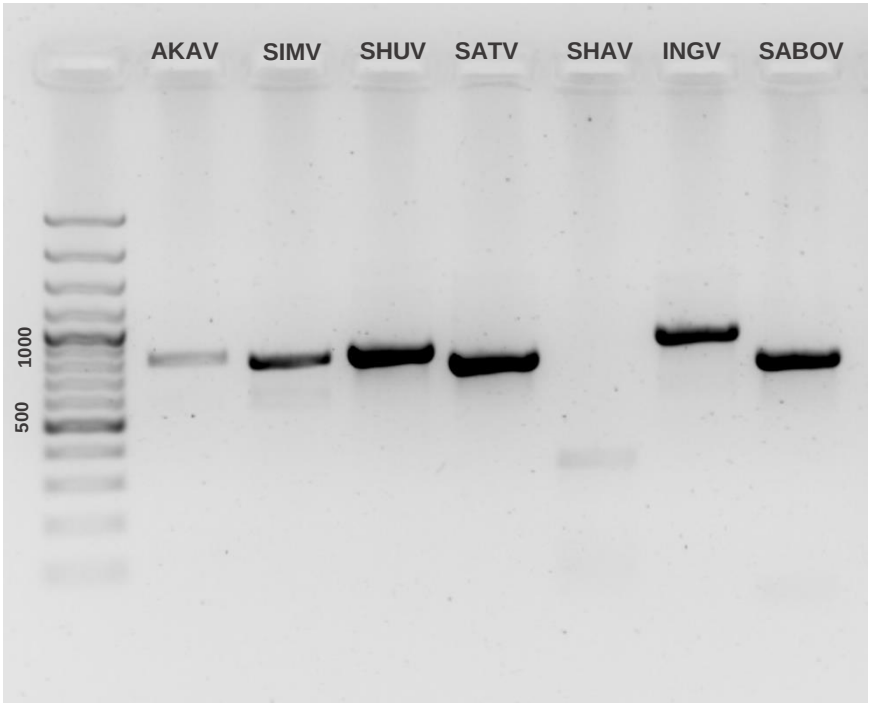
Seven primer sets (Table 6.2) were designed for the full-length sequencing of the DVTD Simbu serogroup isolates's S segment. All primers were able to amplify the targets successfully, i.e. Akabane #2 Vero #3 BHK – 22/5/98, Ar 53 #2 BHK – 2/2/96, Shuni #2 BHK #2 Vero – 22/5/98, Sathuperi #2 BHK – 2/2/96, Ingwavuma #2 BHK – 2/2/96 and Sabo #2 Vero #1 BHK – 3/8/94, with the exception of Shamonda #1 Vero – 21/1/94, where amplification was ineffective (Figure 6.5). Subsequent investigation confirmed that the primer in question (Shamonda_NC018464R) was indeed complementary with the published sequence used for its design (Genbank® accession number NC018464), but targeted a non-virus sequence that had been included in the published sequence. Successful amplification of Shamonda #1 Vero – 21/1/94 was accomplished using the reverse primer utilised for SATV amplification (Sathuperi_HE795104R), as this was 100% identical to the SHAV sequence.

**Table 6.2. Sequencing primers**

| Name | Sequence (5'→3') | Location | Length | Tm (ºC) | GC% |
|---|---|---|---|---|---|
| Akabane_AB289319F | GAACTCCACTATTAACTACGCATTG | 7-32 | 25 | 62 | 40 |
| Akabane_AB289319R | AAAGGTGTGCACCACATAGA | 785-805 | 20 | 62 | 45 |
| Simbu_ NC_018477F | AATGGCAAACCAATTCA | 24-41 | 17 | 55 | 35 |
| Simbu_ NC_018477R | GGCGTACAACACATAGA | 787-804 | 17 | 56 | 47 |
| Shuni_KU937313F | AGTGTAYTCCACTATAGAACAAAGC | 5-30 | 25 | 62 | 40 |
| Shuni_KU937313R | AGTGTGCTCCACATAGAACAAT | 828-850 | 22 | 62 | 41 |
| Sathuperi_HE795104F | CACTACTGAAATATGTCAAGCCAATTC | 32-59 | 27 | 63 | 37 |
| Sathuperi_HE795104R | CTCAACAGAAGCCTTGCAGTAT | 805-827 | 22 | 63 | 46 |
| Shamonda_NC018464F | CCACTATTAACTACAGAAATATGTCAAGCC | 11-41 | 30 | 64 | 37 |
| Shamonda_NC018464R | GGACCCGAAAGATGGTGAACTA | 848-870 | 22 | 64 | 50 |
| Ingwavuma_KF697141F | AGTAGTGTACTCCAC**W**ATTCAA | 1-23 | 22 | 59 | 36 |
| Ingwavuma_KF697141R | GTAGTGTGCTCCCAATTCA | 955-974 | 19 | 59 | 48 |
| Sabo_AF362396F | GTGTACTCCACTATTAACTACGTACC | 5-31 | 26 | 62 | 42 |
| Sabo_AF362396R | GAATTGGCGTGTCTCACATAGA | 791-813 | 22 | 62 | 45 |

**Tm – melting temperature; GC% - guanine-cytosine content**

**Figure 6.5. Agarose gel electrophoresis**




**The gel shows a 100 bp DNA ladder in the first well, successful amplification of AKAV, SIMV, SHUV, SATV, INGV and SABOV and no amplification of SHAV.**

### *6.3.2.2. Real-time RT-PCR primers and probes*

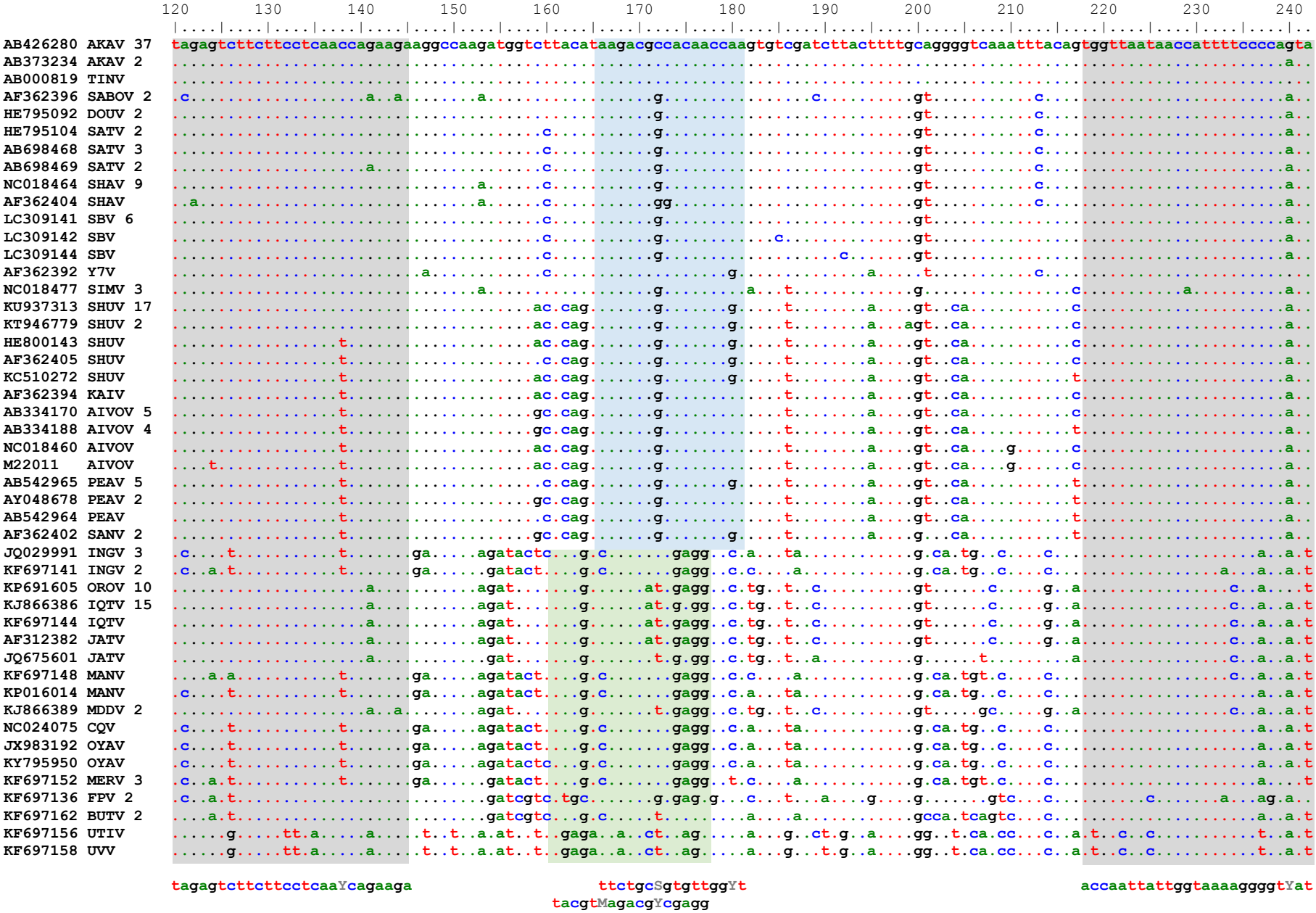
A conserved region in sequence coding the N protein was identified and selected for the development of a group-specific real-time RT-PCR for the detection of Simbu serogroup viruses. A single set of primers was designed, based on the published sequencing data of twenty-eight out of thirty-two Simbu serogroup members, in order to amplify a product of 122bp. Two TaqMan® MGB probes labelled with different dyes were developed to allow for the distinction between phylogenetic clades. Both primer and probe sets are degenerate, as this genetically diverse group of viruses presented multiple nucleotide mismatches after alignment (Table 6.3 and Figure 6.6).

**Table 6.3. Group-specific real-time RT-PCR primers and probes**

| Name | Sequence (5'→ 3') | Location | Length | Tm (°C) | GC% |
|---|---|---|---|---|---|
| Simbu_F | TAGAGTCTTCTTCCTCAA**Y**CAGAAGA | 120-145 | 26 | 57 | 40 |
| Simbu_R | TA**Y**TGGGGAAAATGGTTATTAACCA | 218-242 | 25 | 58.6 | 34 |
| Simbu_CladeAP | VIC®-TACGT**M**AGACG**Y**CGAGG-MGB | 161-177 | 17 | 67 | 59 |
| Simbu_CladeBP | FAM™-T**Y**GGTTGTG**S**CGTCTT-MGB | 166-181 | 16 | 69 | 53 |

**Tm – melting temperature; GC% - guanine-cytosine content**

## Figure 6.6. Simbu serogroup sequence alignment




**Genome region (120-242) targeted by the group-specific real-time RT-PCR, showing the nucleotide variation within the S segment. Numbers following the sequence identification indicate the number of identical sequences available in GenBank®. Dots indicate identity with the first sequence. Primers are represented by the grey shades, probe Simbu_CladeBP is represented by the blue shade (for Akabane, Tinaroo, Sabo, Douglas, Sathuperi, Shamonda, Schmallenberg, Yaba-7, Simbu, Shuni, Kaikalur, Aino, Peaton and Sango viruses detection) and probe Simbu_CladeAP is represented by the green shade (for Ingwavuma, Oropouche, Perdões, Iquitos, Jatobal, Manzanilla, Madre de Dios, Cat Que, Oya, Mermet, Facey's Paddock, Buttonwillow, Utinga and Utive viruses detection). Primer and probe sequences are indicated in the bottom.**

## 6.4. Sequencing

The S segment of the DVTD's Simbu serogroup isolates, AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV, which represent the prototype strains, Ja Gar 39, SA Ar 53, An 10107, IG 10310, An 5550, SA An 4165 and An 9398 respectively, as well as two other South African strains of SHUV and SABOV, 8912 and Cu 1/70 respectively, were sequenced for the first time and the ORFs compared with published data. AKAV showed 5% nucleotide variation relative to Ja Gar 39 (GenBank® accession numbers AF034939 and AB000852). SIMV was 100% identical to SA Ar 53 (NC018477) and varied 0.3% to (HE795110). The two sequenced strains of SHUV, An 10107 and 8912, were identical to each other and varied by 0.99% when compared to the prototype strain (HE800143). SHAV was 100% identical to An 5550 (NC018464 and HE795107). INGV varied 0.14% compared to SA An 4165 (KF697141). SABOV strain An 9398 was 100% identical to a published sequence (HE795098) and varied by 0.14% to AF362396, and 1.7% to the other sequenced strain Cu 1/70. No reference to SATV strain IG 10310 sequence was found in GenBank®, but was 100% identical to two sequences (NC018462 and HE795104) published by Goller and colleagues (Goller *et al.*, 2012).

## 6.5. The group-specific RT-qPCR assay

Only a few real-time RT-PCR assays have been developed for the molecular diagnosis of Simbu serogroup orthobunyaviruses, describing either virus-specific approaches in a simplex format (Bilk *et al.*, 2012; Van Eeden *et al.*, 2014b; Tauscher *et al.*, 2017), or broader approaches in duplex (Stram *et al.*, 2004b) or multiplex formats (Fischer *et al.*, 2013; Lee *et al.*, 2015; Shirafuji *et al.*, 2015; Naveca *et al.*, 2017). The majority of the assays target the S segment, which has the least amount of variation (Figure 6.3) (Saeed *et al.*, 2000; Nunes *et al.*, 2005; Acrani *et al.*, 2010; Vasconcelos *et al.*, 2011; Hang *et al.*, 2014; Cardoso *et al.*, 2015), but an assay targeting the L segment has also been described (Fischer *et al.*, 2013). The published methods describing the broader detection capacity of the Simbu serogroup viruses utilise either a SYBR® Green based chemistry able to recognise viruses from both clades, which is not absolutely specific (Fischer *et al.*, 2013), or a TaqMan® based chemistry that recognises only clade B viruses (Shirafuji *et al.*, 2015).

In this dissertation, a group-specific TaqMan® based real-time RT-PCR is described, targeting the S segment, and designed to distinguish viruses from both phylogenetic clades.

## 6.5.1. Development

### *6.5.1.1. Pilot run*

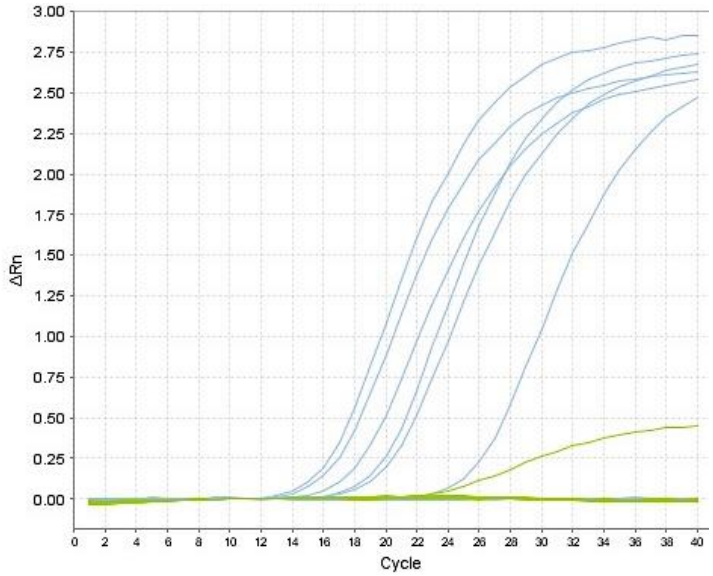
The group-specific RT-qPCR detected all the tested Simbu serogroup isolates. The quantification cycles ($C_q$) values are shown in Table 6.4. No cross-reactivity was observed between the two different probes, which allow distinction between the two clades. Nevertheless, performance of both probes was quite distinct with probe Simbu_CladeAP showing much lower fluorescence intensity (Figure 6.7).

**Table 6.4. Pilot run $C_q$ values**

| Virus | $C_q$ value |
|---|---|
| AKAV | 15.5 |
| SIMV | 15.0 |
| SHUV | 18.5 |
| SATV | 17.0 |
| SHAV | 18.9 |
| INGV | 25.7 |
| SABOV | 24.7 |

**$C_q$ – quantification cycle**

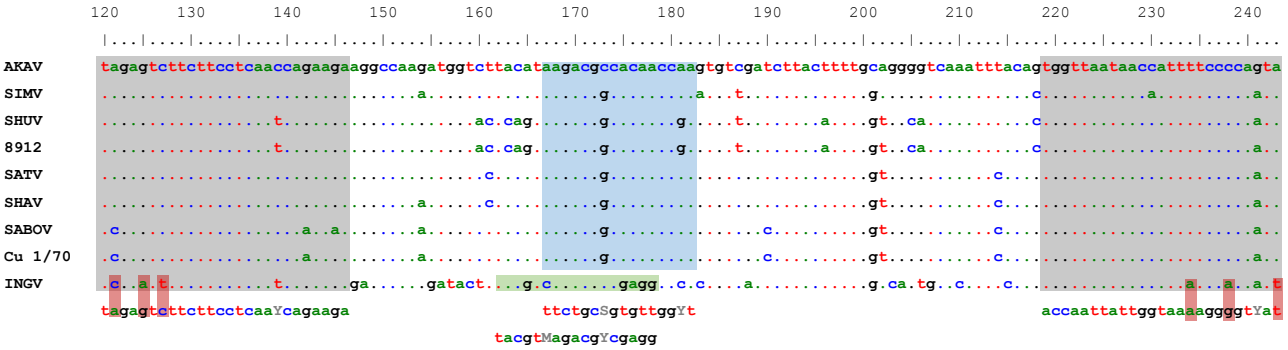
**Figure 6.7. Pilot run amplification plot**




**Amplification curves of the group-speciifc real-time RT-PCR plotted as fluorescence intensity (ΔRn) against cycle number showing successful amplification of AKAV, SIMV, SHUV, SATV, SHAV, SABOV (blue curves), and INGV (green curve). Negative control is represented by the flat line.**

The difference obtained in terms of fluorescence intensity may be explained with the amplification process itself, as multiple nucleotide mismatches were found between both primers and the clade A isolate stored at DVTD, Ingwavuma #2 BHK – 2/2/96, at both primers's 5' end (Figure 6.8).

**Figure 6.8. DVTD's isolates sequence alignment**




**Note the multiple nucleotide mismatches between the primers and INGV sequence which may explain the poor amplification performance (red rectangles).**

## *6.5.1.2. Comparison with a pan-Simbu assay (Fischer et al., 2013)*

In 2013, Fischer and colleagues published a real-time RT-PCR experiment for the broad-range detection of Simbu serogroup viruses, using fourteen out of the thirty-two members, which was named pan-Simbu. This intercalating dye based qPCR assay was at the time of publication the first available tool for the broad screening of Simbu viruses and was able to detect all the tested viruses, which included AINOV, AKAV, DOUV, PEAV, SABOV, SANV, SATV, SHAV, SHUV, SIMV, THIV, TINV and SBV from clade B and OROV from clade A. However, this system is not specific for Simbu serogroup viruses, as it was also able to detect Bunyamwera serogroup orthobunyaviruses.

The pan-Simbu was reproduced at DVTD and again allowed the detection of all the tested viruses, AKAV, SIMV, SHUV, SATV, SHAV and SABOV from clade B and INGV from clade A. Nevertheless, using the same samples, the novel group-specific RT-qPCR showed much higher sensitivity (Table 6.5).

**Table 6.5. Comparison with the pan-Simbu**

| Virus | $C_q$ group-specific | $C_q$ pan-Simbu | $C_q$ Difference | Fold Difference* |
|---|---|---|---|---|
| AKAV $10^{-1}$ | 17.61 | 32.13 | 14.52 | 23496 |
| SIMV $10^{-1}$ | 16.49 | 27.76 | 11.27 | 2465 |
| SHUV $10^{-1}$ | 19.14 | 33.37 | 14.23 | 19175 |
| SATV $10^{-1}$ | 27.91 | 33.75 | 5.84 | 57 |
| SHAV $10^{-1}$ | 21.52 | 28.75 | 7.23 | 150 |
| INGV $10^{-1}$ | 22.46 | 36.91 | 14.45 | 22431 |
| SABOV $10^{-1}$ | 26.79 | 32.21 | 5.41 | 43 |

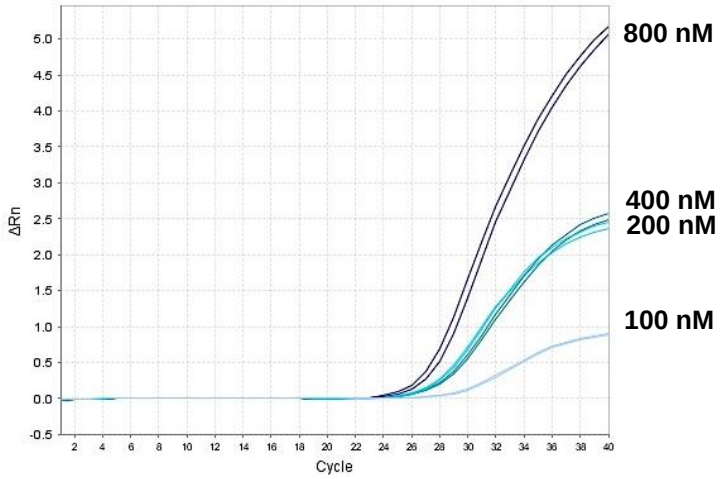
**The table shows the quantification cycle ($C_q$) values obtained with the novel group-specific RT-qPCR and with the pan-Simbu using a 1:10 dilution of tissue culture viruses. * An efficiency of 100% has been assumed - theoretically, the group-specific RT-qPCR would be able to detect a 1:23496 dilution of AKAV $10^{-1}$ resulting in the same $C_q$ value obtained with the pan-Simbu.**

## 6.5.2. Optimisation of primer and probe concentrations

Optimal concentration was defined as the lowest concentration of primers or probe able to generate a low $C_q$ with high efficiency.

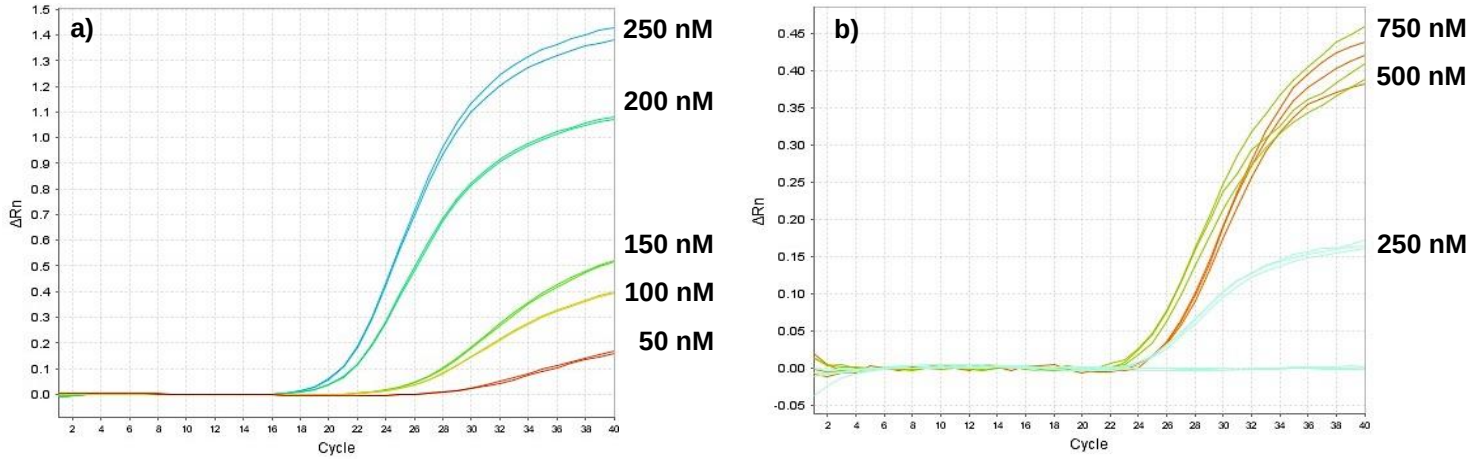
The lowest $C_q$ was obtained when using 800 nM primer concentration and no significant difference was observed between a 200 nM or 400 nM primer concentration (Figure 6.9). The detection of the target improved as the concentration of the probe increased. To limit the primer/probe concentrations in order to allow multiplex detection, a final primer concentration of 400 nM, a probe Simbu_CladeBP concentration of 250 nM and a probe Simbu_CladeAP concentration of 500 nM probe in the reaction was selected (Figure 6.10).

**Figure 6.9. Amplification plot obtained for primer optimisation**




**SABOV amplification plot during the optimisation of primer concentration. The probe Simbu_CladeBP concentration in the reactions was 250 nM.**

**Figure 6.10. Amplification plots obtained for probe optimisation**




**SATV (a) and INGV (b) amplification plots during the optimisation of probe Simbu_CladeBP and Simbu_CladeAP concentration, respectively. The primer concentration in the reactions was 200 nM.**

## 6.5.3. Efficiency

Amplification efficiency is defined as the fraction of target molecules that are generated per PCR cycle (Lalam, 2006; Alvarez, Vila-Ortiz, Salibe, Podhajcer, & Pitossi, 2007), or in other words, a measure of the rate at which the DNA polymerase produces an amplicon (Tenreiro *et al.*, 2014). Therefore, an efficiency of 100% represents the duplication of the number of amplification product per cycle, although is rarely equal to a hundred and may decrease as the reaction progresses with the decline of reagents (Mehra & Hu, 2005).

The efficiency was calculated from the linear regression between the $C_q$ and $TCID_{50}$/reaction values obtained for each isolate (Table 6.6 and Figure 6.11).

**Table 6.6. Amplification efficiency of a group-specific Simbu serogroup *Orthobunyavirus* assay.**

| Virus | Efficiency (%) |
|---|---|
| AKAV | 99 |
| SIMV | 96 |
| SHUV | 96 |
| SATV | 97 |
| SHAV | 84 |
| INGV | 93 |
| SABOV | 110 |

Efficiency values of less than 90% may be caused by *Taq* DNA polymerase contamination with inhibitors, inappropriate $T_M$ or poorly design primers, whereas efficiency values greater than 100% usually result from contamination with non-specifiic products or primer dimers (Tenreiro *et al.*, 2014).

## 6.5.4. Sensitivity

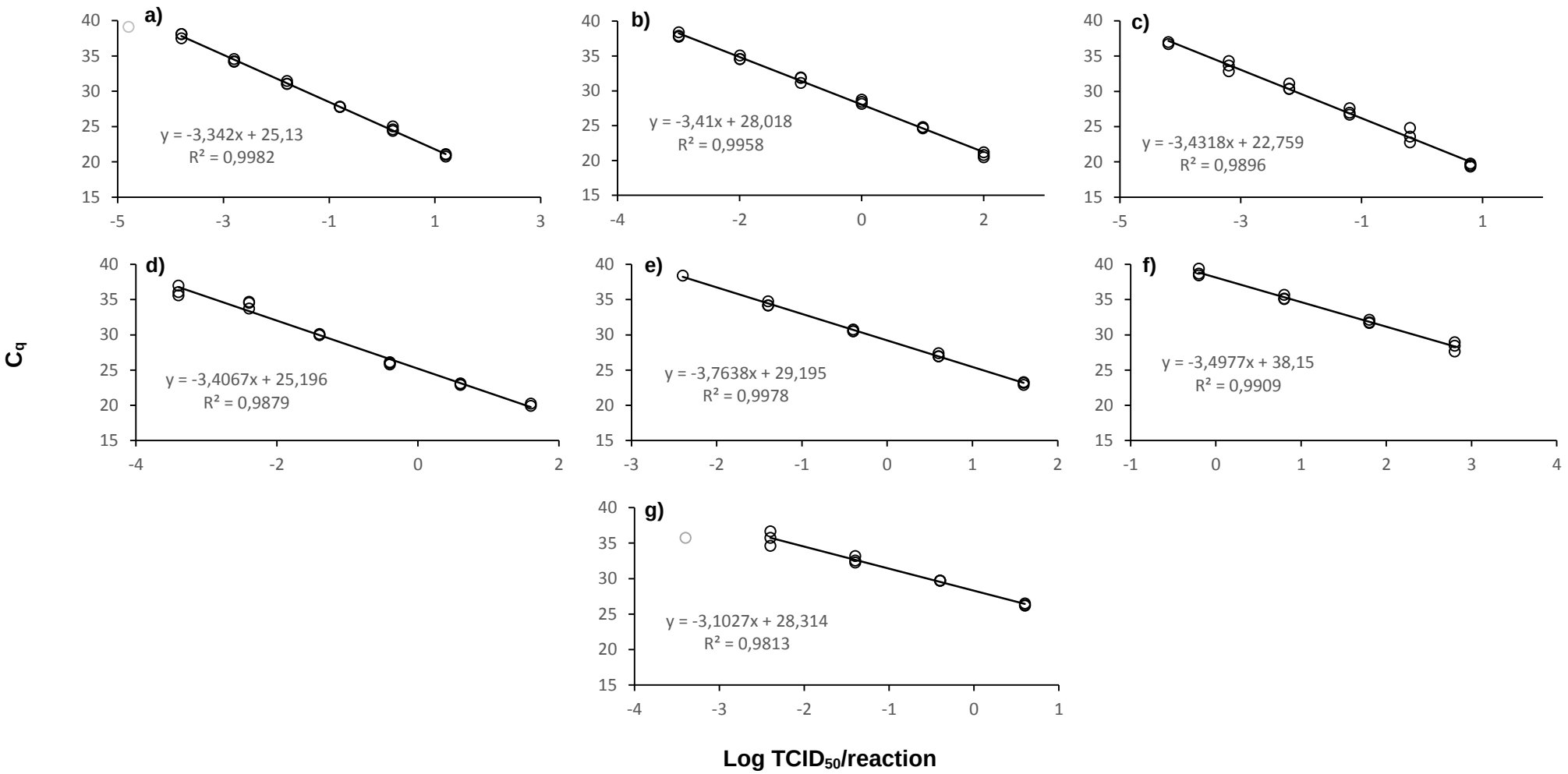
The 95% LOD of the assay, expressed by $TCID_{50}$/reaction, was determined by probit analysis for each virus (Figure 6.12). The LOD, 95% confidence intervals, as well as the correspondent $C_q$ values are shown in **Erro! A origem da referência não foi encontrada.**.

**Table 6.7. The 95% limit of detection of a group-specific Simbu serogroup *Orthobunyavirus* assay.**

| Virus | $TCID_{50}$/reaction | 95% confidence interval | $C_q$ value |
|---|---|---|---|
| AKAV | $10^{-3,61}$ | $10^{-3,98}$ to $10^{-2,38}$ | 37.21 |
| SIMV | $10^{-2,38}$ | $10^{-2,94}$ to $10^{-0,18}$ | 36.12 |
| SHUV | $10^{-3,42}$ | $10^{-3,66}$ to $10^{-2,05}$ | 34.50 |
| SATV | $10^{-3,32}$ | $10^{-3,56}$ to $10^{-1,95}$ | 36.51 |
| SHAV | $10^{-1,67}$ | $10^{-2,07}$ to $10^{1,57}$ | 35.49 |
| INGV | $10^{0,39}$ | $10^{0,03}$ to $10^{1,56}$ | 36.80 |
| SABOV | $10^{-2,70}$ | $10^{-3,01}$ to $10^{-1,59}$ | 36.69 |

TCID – tissue culture infectious dose; $C_q$ – quantification cycle

**Figure 6.11. Linear regression analysis for efficiency calculation**




**Efficiencies of a group-specific Simbu *Orthobunyavirus* assay, determined by regression analysis of a ten-fold dilution series of tissue cultured AKAV (a), SIMV (b), SHUV (c), SATV (d), SHAV (e), INGV (f) and SABOV (g). Each dilution had three replicates (black circles). Outliers were not included in the calculation (grey circles).**

## 6.5.5. Specificity

The assay was shown to be specific for the detection of Simbu serogroup orthobunyaviruses, as no cross-reactions were observed *in vitro* with BVDV-1, BoHV-1, BTV-1, BEFV, RVFV, WSLV or PALV. *In silico* specificity analysis showed no cross-reactivity with other genetically related viruses which may be differential diagnosis along with Simbu serogroup viruses, namely those belonging to the Bunyamwera serogroup orthobunyaviruses that also can cause abortion and teratology in ruminants (Singh & Pavri, 1966; Chung, Livingston, Edwards, Crandell, Shope, Shelton, & Collisson, 1990; de la Concha-Bermejillo, 2003).

## 6.5.6. Repeatability

Intra and inter-run standard seviation (SD) as well as coefficient of variation (CV) values for both probe Simbu_CladeAP and probe Simbu_CladeBP detection are shown in Table 6.8 and Table 6.9, respectively. Intra-run SD ranged from 0.50 and 0.71 for probe Simbu_CladeAP and 0.14 to 0.68 for probe Simbu_CladeBP. The inter-run SD ranged from 0.19 to 0.60 for probe Simbu_CladeAP and from 0.31 to 0.97 for probe Simbu_CladeBP. CV ranged from 1.36% and 2.01% for probe Simbu_CladeAP and 0.63% and 2.91% for probe Simbu_CladeBP detection.

**Table 6.8. Intra and Inter-run variation for probe Simbu_CladeAP detection**

| LogTCID$_{50}$/rxn | $C_q$ Total Mean | $C_q$ Intra-run SD | $C_q$ Inter-run SD | $C_q$ Total SD | CV (%) |
|---|---|---|---|---|---|
| 0.80 | 34.56 | 0.56 | 0.35 | 0.67 | 1.93 |
| 0.50 | 36.57 | 0.50 | 0.19 | 0.50 | 1.36 |
| 0.20 | 37.68 | 0.56 | 0.59 | 0.75 | 2.00 |
| -0.10 | 38.50 | 0.71 | 0.60 | 0.77 | 2.01 |
| -0.40 | 39.03 | 0.59 | 0.55 | 0.62 | 1.59 |

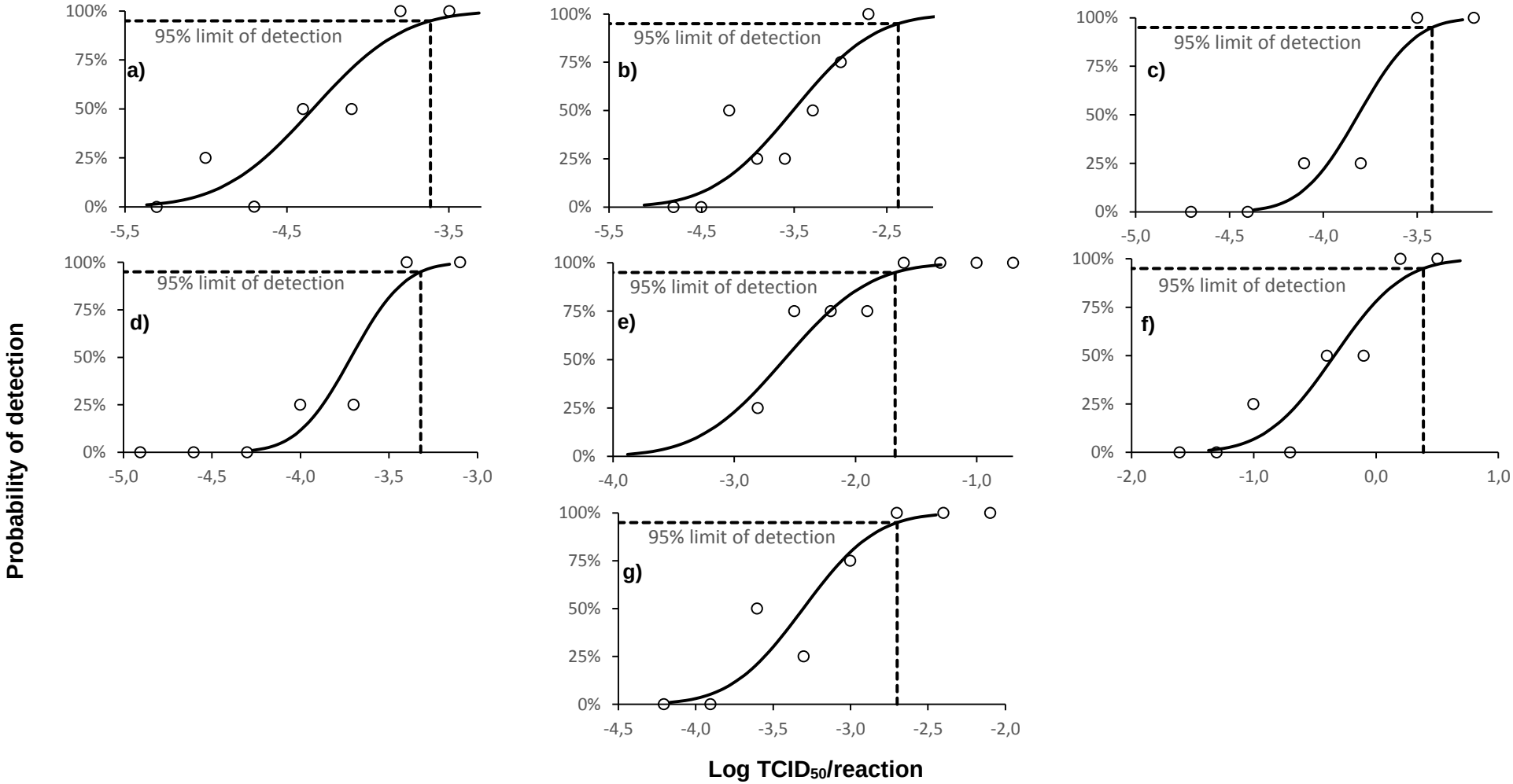
**TCID – tissue culture infectious dose; Rxn – reaction; $C_q$ – quantification cycle; SD – standard deviation; CV – coefficient of variation**

**Table 6.9. Intra and Inter-run variation for probe Simbu_CladeBP detection**

| LogTCID$_{50}$/rxn | $C_q$ Total Mean | $C_q$ Intra-run SD | $C_q$ Inter-run SD | $C_q$ Total SD | CV (%) |
|---|---|---|---|---|---|
| -1.00 | 33.86 | 0.31 | 0.68 | 0.67 | 1.99 |
| -1.30 | 34.88 | 0.38 | 0.49 | 0.57 | 1.64 |
| -1.60 | 36.45 | 0.45 | 0.47 | 0.58 | 1.59 |
| -1.90 | 37.65 | 0.68 | 0.97 | 1.10 | 2.91 |
| -2.20 | 38.59 | 0.51 | 0.36 | 0.62 | 1.62 |
| -2.50 | 39.50 | 0.14 | 0.31 | 0.25 | 0.63 |

**TCID – tissue culture infectious dose; Rxn – reaction; $C_q$ – quantification cycle; SD – standard deviation; CV – coefficient of variation**

**Figure 6.12. 95% limit of detection of the assay**



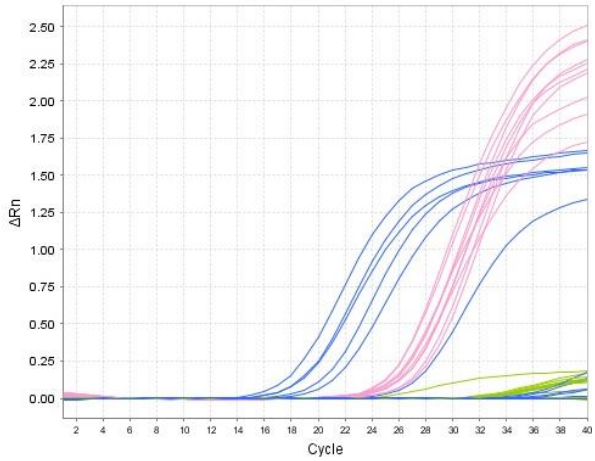

**A two-fold dilution series of $10^{-5}$ AKAV (a), $10^{-5}$ SIMV (b), $10^{-5}$ SHUV (c), $10^{-5}$ SATV (d), $10^{-3}$ SHAV (e), $10^{-3}$ INGV (f) and $10^{-3}$ SABOV (g). A circle represents four replicates of each dilution. The dashed line indicates the 95% limit of detection ($TCID_{50}$/reaction,) which is a) $10^{-3,61}$ for AKAV, b) $10^{-2,38}$ for SIMV, c) $10^{-3,42}$ for SHUV, d) $10^{-3,32}$ for SATV, e) $10^{-1,67}$ for SHAV, f) $10^{0,39}$ for INGV and g) $10^{-2,70}$ for SABOV.**

## 6.5.7. Testing of field samples

### *6.5.7.1. Abortion products from Namibia*

Simbu serogroup members have been identified as cause of abortion in ruminants over the years (Inaba, Kurogi, & Omori, 1975; Coverdale *et al.*, 1978; Parsonson & McPhee, 1985; St. George & Kirkland, 2004; Steinrigl *et al.*, 2014; Wernike & Beer, 2017). Regardless, the pooled organs and brains from the five Namibian bovines with the abortion syndrome of unkown aetiology tested negative for Simbu serogroup viruses. The nucleic acid purification system worked well, as the control of extraction (bovine β-actin) tested as expected. Non-specific amplification occurred, as a background signal similar to the negative control arised. Fluorescence artifacts (Caraguel, Stryhn, Gagne, Dohoo, & Hammell, 2011), nucleic acid contamination from elsewhere in the laboratory or carryover contamination from amplification products and primers used in prior PCR experiments (Longo, Berninger, & Hartley, 1990) are common causes of false positive results in PCR (Kwok & Higuchi, 1989) and may explain the non-specific amplification signal (Figure 6.13).

**Figure 6.13. Amplification plot of the Namibian samples**



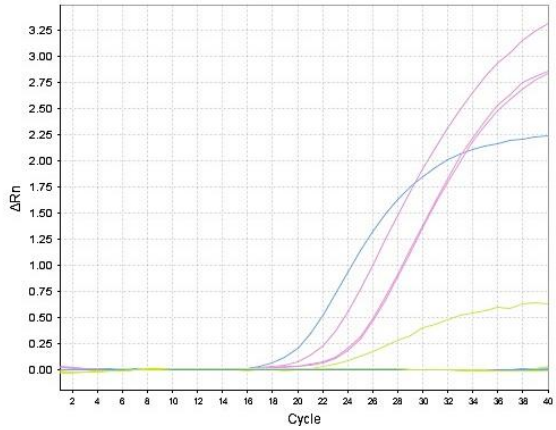

**qPCR plot showing amplification of Simbu serogroup positive controls (blue curves), β-actin internal controls (pink curves) as well as non-specific products (green and blue curves on the right bottom corner).**

### *6.5.7.2. Calf with neurological signs*

Simbu serogroup orthobunyaviruses have been associated with encephalomyelitis among other CNS pathologies in domestic animals (Miyazato *et al.*, 1989; Coetzer & Howell, 1998; Tsuda *et al.*, 2004; Kono *et al.*, 2008; Oem *et al.*, 2012; van Eeden *et al.*, 2012). Nevertheless, the tested samples from the calf, namely pooled organs, blood, cortex and cerebellum fragments were negative for the presence of Simbu serogroup viruses. Nucleic acid purification and PCR conditions were validated by bovine β-actin, clade A virus (INGV) and clade B virus (AKAV) positive controls amplification, respectively. (Figure 6.14).

Parallel aetiological investigation confirmed the presence of *Ehrlichia ruminantium* in the capillary endothelium of the calf's brain.

**Figure 6.14. Amplification plot of the calf samples**

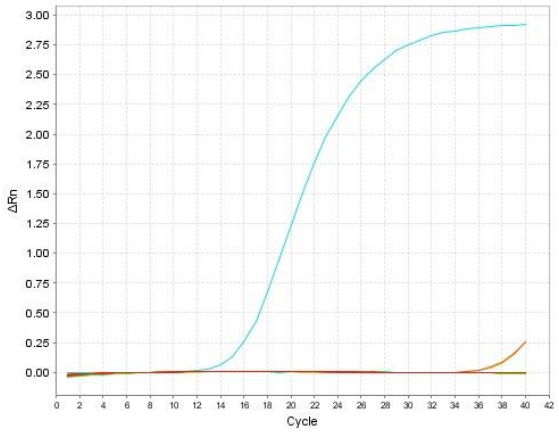



**qPCR plot showing amplification of bovine β-actin (pink curves), AKAV positive control (blue curve) and INGV positive control (green curve). The flat lines represent the calf's pooled organs, blood, cortex and cerebellum as well as the negative control of reaction.**

## *6.5.7.3. Mosquitoes from Bumbe lake*

Almost all Simbu serogroup members are arboviruses, at least fifteen viruses are known to be mosquito-borne, and at least eleven have been isolated in mosquitoes of the genus *Culex.* Two out of fifty different pools of the tested *Culex* pipiens mosquitoes resulted in a late amplification signal ($C_q$ 38.23 and 38.31) (Figure 6.15). It can be assumed that high $C_q$ values are generated by cross contamination or non-specific amplification of background nucleic acids (Longo *et al.*, 1990), even though the no template control tested negative, or in other hand by degradation of the probe fluorophore (Burns & Valdivia, 2008). In order to prevent false positive results, there is a tendency among reseachers to consider as negative any $C_q$ values above a certain arbitrary cut-off, which can be selected e.g. as the $C_q$ value that corresponds to the defined lower limit of detection of the assay (Caraguel *et al.*, 2011).

**Figure 6.15. Amplification plot of the Bumbe mosquitoes**




**qPCR plot showing amplification of a Simbu clade B virus control, SIMV (blue curve) and late amplification of two mosquito pools (orange curves). The flat line represents the negative control of reaction.**

# 7. Conclusion

Overall, this work shows the development of a novel group-specific one-step Taqman®-based RT-qPCR assay, with the ability to differentiate between two phylogenetic clades, which was optimised and laboratory validated to provide a rapid, sensitive and specific molecular diagnostic method for the broad detection of at least thirty-one viral pathogens, of which some have significant relevance in public and animal health.

This assay showed more sensitivity, specificity and broad detection capacity when compared with other published methods for detection of Simbu serogroup viruses. Therefore, it can be a useful diagnostic tool, either in countries where a certain Simbu-associated endemic disease is present or in countries where the disease is exotic and demands a quick identification in case of suspected introduction. The assay can also be useful for the screening of populations of arthropod vectors or wildlife animals, that play an important role in the epidemiology of the Simbu serogroup viruses, thus enabling the development of comprehensive prevalence studies and consequently optimising the management and control of the diseases. The assay may still be useful for the detection of other Simbu serogroup orthobunyaviruses that have yet to be described.

The Simbu serogroup viruses used for the development of the assay, those stored in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Sciences of the University of Pretoria, were for the first time subjected to sequencing. It is also the first time that genomic sequencing data of some of these viruses will be available in a genetic sequence database (GenBank®), namely the prototype strain of SATV, SHUV strain 8912 and SABOV strain Cu 1/70.

A comprehensive review of the Simbu serogroup viruses was carried out, including sites of viral isolation and associated seroconversion. This review makes clear the cosmopolitan distribution of the serogroup members, which is highlighted by the map generated using a geographic information system tool. As new cases are detected, there is always the possibility of updating the map, since there is a lack of information in official sources, e.g. OIE World Animal Health Information System. Thus, this work can be useful for the development of further studies, e.g. cluster analysis, risk analysis or epidemiological modelling.

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# 9. Annex

## Manuscript

**Abstract**

The Simbu serogroup within the genus *Orthobunyavirus* belongs to the family *Peribunyaviridae* and comprises 32 recognised three-segmented negative-sense single-stranded RNA viruses. This group of arthropod-borne viruses, cosmopolitan distributed, infect humans and domestic animals, causing clinical disease. A novel group-specific TaqMan®-based real-time RT-PCR assay was developed, optimized and laboratory validated for the broad detection of the Simbu serogroup orthobunyaviruses. The published genomic data of the Simbu serogroup members was evaluated, and a conserved region, situated in the segment coding the nucleocapsid protein, was selected to design a universal primer set (Simbu_F and Simbu_R) and a pair of differently labelled hydrolysis probes (Simbu_CladeAP and Simbu_CladeBP), which allow the distinction between the two phylogenetic clades of the Simbu serogroup. Seven prototype Simbu serogroup isolates were used for the development of the assay, namely *Akabane orthobunyavirus* (AKAV), *Simbu orthobunyavirus* (SIMV), *Shuni orthobunyavirus* (SHUV), *Sathuperi orthobunyavirus* (SATV), *Shamonda orthobunyavirus* (SHAV), Ingwavuma virus (INGV) and Sabo virus (SABOV). The primer and probe concentrations in the PCR reactions were optimized. The efficiency of the assay was determined for each one of the viruses: AKAV (99%), SIMV (96%), SHUV (96%), SATV (97%), SHAV (84%), INGV (93%) and SABOV (110%). To assess the specificity of the assay, a panel constituted of genetically related, causative agents of abortion in ruminants and arthropod-borne viruses was selected for *in vitro* analysis, and *in silico* analysis was also performed. The assay was shown to be specific, as no cross-reactions were observed either *in vitro* or *in silico* and sensitive, with a 95% limit of detection ranging from $10^{0,39}$ to $10^{-3,61}$ $TCID_{50}$/reaction, for the detection of Simbu serogroup viruses. The repeatability of the assay was evaluated for both probes detection. The repeatability of the assay was evaluated for both probes detection, using the intra-run standard deviation (0.50 - 0.71 for probe Simbu_CladeAP and 0.14 - 0.68 for probe Simbu_CladeBP), inter-run standard deviation (0.19 - 0.60 for probe Simbu_CladeAP and 0.31 - 0,97 for probe Simbu_CladeBP) and coefficient of variation (1.36% - 2.01% for probe Simbu_CladeAP and 0.63% - 2.91% for probe Simbu_CladeBP).

**Introduction**

The Simbu serogroup within the genus *Orthobunyavirus* belongs to the family *Peribunyaviridae* (Adams *et al.*, 2017) and comprises 32 recognised three-segmented negative-sense single-stranded RNA viruses (Saeed *et al.*, 2001a; Elliott & Blakqori, 2011; Ladner *et al.*, 2014; Tilston-Lunel *et al.*, 2015). This group of arthropod-borne viruses (arboviruses) is known to cause central nervous system (CNS) disease in humans (Anderson *et al.*, 1961; Aguilar *et al.*, 2011; Ladner *et al.*, 2014) and reproductive system and CNS disease in domestic and livestock species (Coverdale *et al.*, 1978; Charles, 1994; Coetzer & Howell, 1998; Hoffmann *et al.*, 2012; van Eeden *et al.*, 2012; Hirashima *et al.*, 2017). These agents have been isolated from a wide range of wild mammals and birds (Anderson *et al.*, 1960; McIntosh *et al.*, 1965; Calisher *et al.*, 1969; Reeves *et al.*, 1970; Carey *et al.*, 1971; Pajot, 1980; Seymour *et al.*, 1983; Navarro *et al.*, 2016). The viruses in the

serogroup have a cosmopolitan distribution and have been divided phylogenetically into two different clades, designated A, those associated with clinical disease in humans, and B, associated with abortion and teratology in ruminants, as well as neurologic disease in horses (Ladner *et al.*, 2014). Rapid viral detection and large population screening capability provide important epidemiological data, enhance the surveillance and control of emerging, reemerging or novel diseases and consequently optimize the management in terms of public and animal health (Richman *et al.*, 1984). Culture-based and serologic methods play a pivotal role in the diagnostic of viral infections but can be time-consuming and technically demanding, thus limiting its usefulness when rapid diagnosis is needed, and technically demanding, further requiring skilled and experienced personnel as well as adequate facilities to manipulate cell lines or pathogenic viruses (Souf, 2016). The use of molecular technologies based on nucleic acid amplification, such as conventional or real-time PCR, has increased to the point where it is now considered as the gold standard for viral diagnostics (Mackay *et al.*, 2002). In comparison with the conventional technique, although the cost of equipment and reagents are substantially higher, real-time PCR has greater analytical sensitivity and specificity, is quicker to perform, has multiplex capacitiy and allow viral genome quantification, being the most widely used method for direct virus detection (Mackay *et al.*, 2002; Wernike & Beer, 2017). A few laboratory techniques for the broad detection of Simbu serogroup viruses have been described, based on serology, which in fact led to its original designation as serogroup (Kinney & Calisher, 1981), or on molecular techniques. Real-time PCR assays with broad detection capacity have been described, and utilise an intercalated dye chemistry for the detection of both phylogenetic clades (Fischer *et al.*, 2013) or Taqman® chemistry for the detection of clade B viruses (Shirafuji *et al.*, 2015). In this paper, a novel real-time RT-PCR assay is described, utilising Taqman® based chemistry for the broad detection of Simbu serogroup viruses, which allows for the distinction between both clades, based on fluorescence emission spectral discrimination. The development of the real-time RT-PCR assay from the evaluation of all Simbu serogroup viruses's published genomic data, in order to design specific primers and probes, including the laboratory characterization in terms of efficiency, sensitivity, specificity and repeatability, is described.

## Materials and methods

### Simbu serogroup isolates

The Simbu serogroup orthobunyaviruses stored at the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Sciences, University of Pretoria were used for the development and optimization of the assay (Table 1). All the isolates were identified originally by serology using CF and SNT (Costa Mendes, 1984). The viruses were tested and confirmed positive using a pan-Simbu real-time RT-PCR (Fischer *et al.*, 2013).

### Infectivity

African green monkey kidney (Vero) cells (ATCC®) were grown in minimum essential medium (MEM) with Earle's balanced salts, L-glutamine and non-essential amino acids (Biowest), supplemented with 2,95% tryptose phosphate broth (TPB) (BD Biosciences), 5% γ-irradiated fetal bovine serum (FBS) (Biowest) and 50 mg/ml gentamicin (Virbac) at 37ºC in a humidified 5% $CO_2$ atmosphere. The Vero cell suspension was counted in a Neubauer chamber, adjusted to the optimal concentration of $48\times10^4$ cells/ml and plated (80 µl) in flat-bottomed 96-well clear polystyrene plates (ThermoFisher). A $\log_{10}$ dilution series was carried out for

AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV in MEM with 5% FBS and gentamicin. Tenfold dilutions of the viruses mentioned above ($10^{-1}$ up to $10^{-8}$) were inoculated (100 µl) in the plates (five replicates per dilution) and the expected cytopathic effect (CPE) was read five and six days after inoculation using an inverted optical microscope. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated using the Spearman-Karber method (Spearman, 1908; Kärber, 1931).

**Evaluation of genome variation**

Nucleotide sequences from the three segments of all the Simbu serogroup orthobunyaviruses available on the National Centre for Biotechnology Information's GenBank® were aligned using MAFFT version 7.3.1.3 (Katoh *et al.*, 2017). BioEdit Sequence Alignment Editor version 7.2.3 (Hall, 1999) was used to edit the sequences and to visualize conserved regions present in the ORF. The nucleotide sequences were then translated into amino acids and aligned for a second time to ensure a better alignment quality. EMBOSS: tranalign (Rice *et al.*, 2000) was used to back translate the nucleic acid sequence from the aligned protein. DAMBE software package version 5.3.48 (Xia, 2013) was used to identify identical sequences. To summarize the results and simplify the observation of the nucleotide variation among the three segments, a variation score was calculated using the equation below and used to create a plot.

$$Variation = 1 - x_1 + x_2 + 2x_3 + 3x_4$$

$x_i$ – Frequency of the most common nucleotides; ($1 \leq i \leq 4$)

**Primer and probe design**

**Sequencing primers**

Seven primer sets were designed using the PrimerQuest® tool (Integrated DNA Technologies, Inc.) for the full-length sequencing of the S segment of AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV. The primers were synthesized by Integrated DNA Technologies, Inc., delivered dried, re-suspended in Tris-EDTA buffer (TE buffer) to a 100 µM stock concentration and working aliquots at 20 µM were produced by dilution with ultrapure 18.2 MΩ.cm 25°C water (Elix® Essential 5 and Synergy® water purification systems, Merck)..

**Real-time RT-PCR primers and probes**

After determining the nucleotide variation within the genome, a conserved region situated between the nucleotide positions 120-242 of the S segment was elected to design a serogroup-specific universal primer set, Simbu_F and Simbu_R, targeting all the Simbu orthobunyaviruses with available sequence data. Due to the nucleotide distinctiveness between phylogenetic clades, two different TaqMan® MGB probes (Applied Biosystems®) were designed, one targeting clade A viruses, Simbu_CladeAP, labeled with a VIC® fluorescent dye, and another targeting clade B viruses, Simbu_CladeBP, labeled with a FAM™ (6-carboxyfluorescein) fluorescent dye. Primer Express® version 2.0 (Applied Biosystems®) was used to ensure optimal values in terms of GC-content, $T_M$ and to avoid the formation of secondary structures. *In silico* specificity screening was carried out using Basic Local Alignment Search Tool (BLAST) to prevent non-specific reactions. The primer set and MGB probes were synthesized by Integrated DNA Technologies, Inc. and ThermoFisher Scientific, Inc., respectively. The TE buffer-re-suspended primers and the 100 µM stock probe were both aliquoted to a 20 µM working concentration, by dilution with ultrapure 18.2 MΩ.cm 25°C water (Elix® Essential 5 and Synergy® water purification systems, Merck)..

**Nucleic acid purification**

The viral RNA from the DVTD's isolates was extracted with a magnetic bead based separation method using the KingFisher™ Duo Prime Purification System (ThermoFisher) with the MagMAX™ Pathogen RNA/DNA Kit (Applied Biosystems®), according with the manufacturer's instructions. A standard protocol (MagMAX_Pathogen_Stnd_Vol_DUO.bdz) was used. The nucleic acid was eluted in 50 µl Elution Buffer.

**Sequencing**

The S segments of the isolates stored in DVTD were sequenced for the first time. A conventional PCR was conducted utilising the SuperScript® III One-Step RT-PCR System with Platinum® *Taq* DNA Polymerase protocol (Invitrogen™), which consisted of a 50 µl total reaction volume composed of 25 µl 2X Master Mix, 2 µl SuperScript® III RT/Platinum® *Taq* Mix, 0.5 µl of each 20 µM primer, 2 µl RNA template and nuclease-free water to the final volume. The reactions were performed in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems®) and the reaction conditions were 55ºC for 30 min to reverse transcribe the RNA, 94ºC for 2 min for reverse transcriptase (RT) inactivation, followed by 40 cycles of 94ºC for 15 s (dsDNA denaturation), 55ºC for 30 s (primer annealing) and 68ºC for 1 min (extension). In order to confirm the correct fragment size, gel electrophoresis was carried out with a 2% agarose gel, using 1×Tris-acetate-EDTA (TAE) buffer in the presence of ethidium bromide stain. Five µl GeneRuler 100 bp DNA Ladder, ready-to-use (ThermoFisher) was added to the first well, 1 µl 6× DNA Loading Dye (ThermoFisher) per 5 µl of sample was added to the remaining wells. The gel was electrophoresed at 100 V. To visualise and record the obtained bands, a ChemiDoc™ XRS+ System with Image Lab™ Software version 3.0 (Bio-Rad) was used. The PCR products were purified using the CleanSweep™ PCR purification reagent (Applied Biosystems®) in order to dephosphorylate unincorporated nucleotides and digest unused primers, ensuring accurate downstream sequencing data. The purification protocol was accomplished in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems®) and the conditions were 37ºC for 15 min, followed by 80ºC for 15 min. Sequencing was performed by Inqaba Biotec™ (Pretoria, RSA), using the Sanger method (Sanger *et al.*, 1977) with the same primers used for PCR product amplification. The resultant sequencing data was then analysed, edited and assembled using Pregap4 and Gap4 of the Staden Software Package version 1.5 (Staden *et al.*, 2000).

**The group-specific RT-qPCR assay**

In order to allow the rapid, broad, sensitive and specific detection of the Simbu serogroup orthobunyaviruses, a group-specific TaqMan® based RT-qPCR assay was developed, optimized and validated. All the reactions were set up manually in either MicroAmp™ Optical 8-Cap Strips (ThermoFisher) or MicroAmp™ Fast Optical 96-Well Reaction Plates, 0.1 mL (ThermoFisher) and performed using the TaqMan® Fast Virus 1-Step Master Mix (ThermoFisher) protocol in a StepOnePlus™ Real-Time PCR System (Applied Biosystems®). All the generated data was analysed using the StepOne™ Software version 2.3 (Applied Biosystems®). Reactions were conducted in a 20 µl total volume of which 5 µl were 4×TaqMan® Fast Virus 1-Step Master Mix (MM) and 2 µl of template RNA. Primer, probe and nuclease-free water volumes varied according to the different primer and probe concentrations used for the development and optimisation of the assay and will be specified later. The thermal-cycling conditions comprised a holding stage of 50ºC for 5 min for reverse transcription, 95ºC for 20 s for RT inactivation and initial dsDNA denaturation, followed by 40 cycles of 95ºC for 3 s for denaturation and 60ºC for 30 s for primer annealing and extension.

**Development**

**Pilot run**

A pilot experiment was carried out in order to confirm primer and probe capability and resultant amplification. The RNA from AKAV, SIMV, SHUV, SATV, SHAV and SABOV was used as target for probe Simbu_CladeBP detection and the RNA from INGV as target for probe Simbu_CladeAP detection. The reaction contained 4× TaqMan® Fast Virus 1-Step Master Mix (ThermoFisher), 2 µl RNA, 900 nM of each primer, 250 nM of each probe and nuclease-free water to a final reaction volume of 20 µl.

**Comparison with a pan-Simbu assay (Fischer *et al.*, 2013)**

This group-specific real-time RT-PCR was compared with a pan-Simbu real-time RT-PCR (Fischer *et al.*, 2013). Fischer's assay was conducted with a reverse transcription step using the Omniscript® Reverse Transcription Kit (QIAGEN) in a 20 µl reaction volume, which comprised 2 µl 10× Buffer RT, 2 µl dNTPs mix, 2 µl 10 µM random primers, 1 µl Omniscript® RT, 2 µl template RNA and nuclease-free water. The reaction was run in a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems®) for 60 min at 37ºC. The subsequent step involved a SYBR® Green I based real-time PCR with the KAPA SYBR FAST qPCR Master Mix (2×) ABI Prism™ (Kapa Biosystems) as follows: 10 µl 2× Master Mix, 1 µl 10 µM of each primer (panOBV-L-2959 F and panOBV-L-3274R), 2 µl cDNA and nuclease-free water to a final volume of 20 µl. The real-time reaction was performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems®) and the thermal cycling conditions consisted of a holding stage of 95ºC for 20 s followed by 40 cycles of 95ºC for 3 s, 55ºC for 20 s and 72ºC for 10 s. The melting curve was acquired with the following steps: 95ºC for 15 s, 55ºC for 1 min, data collection with increase of temperature at 0.3%, and 95ºC for 15 s. The comparison was conducted using a $10^{-1}$ dilution of AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV RNA for each experiment.

**Optimisation**

In order to optimize the forward and reverse primer concentration in the RT-qPCR reaction, four different primer concentrations were tested with a constant probe concentration of 250 nM: 100 nM, 200 nM, 400 nM and 800 nM. To optimize the Simbu_CladeAP probe concentration in the reaction, seven different probe concentrations were tested with a constant primer concentration of 200 nM: 50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 500 nM and 750 nM. In addition, a supplementary run was performed using three different primer concentrations, 200 nM, 400 nM and 800 nM against two different probe concentrations, 250 nM and 500 nM. To optimize the Simbu_CladeBP probe concentration in the reaction, five different probe concentrations were tested with a constant primer concentration of 200 nM: 50 nM, 100 nM, 150 nM, 200 nM and 250 nM.

**Efficiency**

The PCR efficiency was determined for the cell cultured AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV. A ten-fold eight-log serial dilution was made for the seven viruses in nuclease-free water and each dilution was tested in triplicate. Concentrations of primers, probe Simbu_CladeAP and probe Simbu_CladeBP in the reactions were 400 nM, 500 nM and 250 nM, respectively. The amplification efficiency was then calculated from the slope of the linear regression between $C_q$ values and log $TCID_{50}$/reaction using the following formula:

$$Efficiency\ (\%) = \left(10^{\left(\frac{-1}{Slope}\right)} - 1\right) \times 100$$

**Sensitivity**

Subsequent to the $\log_{10}$ serial dilution made for efficiency determination, a two-fold dilution series was carried out with $10^{-5}$ AKAV, $10^{-5}$ SIMV, $10^{-5}$ SHUV, $10^{-5}$, $10^{-5}$ SATV, $10^{-3}$ SHAV, $10^{-3}$ INGV and $10^{-3}$ SABOV in nuclease-free water. The dilution series was tested four times in a single run and the results utilized to calculate the 95% limit of detection (LOD) of the assay by probit analysis, using SPSS® Statistics version 25 (IBM®). From this computer-based regression modelling, the analytical sensitivity, which is defined as the viral titer detected 95% of the time, was calculated.

**Specificity**

Nucleic acid of genetically related, causative agents of abortion in ruminants and arthropod-borne viruses such as *pestivirus A* (BVDV-1, V27/04 C #2 MDBK 6.12.04), *bovine alphaherpesvirus 1* (BoHV-1, #3 MDBK 12.10.00, IBR American strain), *bluetongue virus* (BTV-1, pp 28.1.98), *bovine fever ephemerovirus* (BEFV, #2 BHK 20.9.95, Van der Westhuizen strain), *Rift Valley fever phlebovirus* (RVFV, TC50+ #2 Vero 31.5.95)*, wesselsbron virus* (WSLV, #9 Vero 8.6.15) and *palyam virus* (PALV, Kasba #1 Vero 19.2.16) was used to determine the assay specificity. Viral nucleic acid was extracted using the KingFisher™ Duo Prime Purification System (ThermoFisher) with the MagMAX™ Pathogen RNA/DNA Kit (ThermoFisher) according with the manufacturer's instructions and was used as a template along with a clade A and clade B virus control. *In silico* specificity was also performed by BLAST® analysis.

**Repeatability**

The repeatability of the assay was determined by repeating the experiment used for the sensitivity determination four times, in separate runs. INGV and SATV were used as templates to represent Simbu clades A and B, respectively. The data generated were used to assess the intra-run, inter-run and overall variation for both probe Simbu_CladeAP and Simbu_CladeBP detection.

**Results**

**Infectivity**

Six days after viral inoculation, the $TCID_{50}$ was calculated for each isolate. In Vero cell cultures, CPE caused by Simbu serogroup viruses was identified as cytoplasmic shrinking and cell detachment. Log $TCID_{50}$/ml values obtained were 4,9 (AKAV), 5,7 (SIMV), 4,5 (SHUV), 5,3 (SATV), 5,3 (SHAV), 6,5 (INGV) and 4,3 (SABOV).

**Evaluation of the genome variation**

The assessment of the Simbu serogroup members available genomic data showed a high nucleotide variation along the ORF nucleotide sequences of the three segments, S, M and L. Nevertheless, a comparative analysis showed a lower variation score for the S segment, whereas the M segment possessed the highest degree of variation (Figure 1).

**Primer and Probe Design**

**Sequencing primers**

Seven primer sets (Table 2) were designed for the full-length sequencing of the DVTD Simbu serogroup isolates's S segment. All primers were able to amplify the targets successfully, i.e. Akabane #2 Vero #3 BHK – 22/5/98, Ar 53 #2 BHK – 2/2/96, Shuni #2 BHK #2 Vero – 22/5/98, Sathuperi #2 BHK – 2/2/96, Ingwavuma #2 BHK – 2/2/96 and Sabo #2 Vero #1 BHK – 3/8/94, with the exception of Shamonda #1 Vero – 21/1/94, where amplification was ineffective). Subsequent investigation confirmed that the primer in question (Shamonda_NC018464R) was indeed complementary with the published sequence used for its design (Genbank® accession number NC018464), but targeted a non-virus sequence that had been included in the published sequence. Successful amplification of Shamonda #1 Vero – 21/1/94 amplification was accomplished using the reverse primer utilized for SATV amplification (Sathuperi_HE795104R), as this was 100% identical to the SHAV sequence.

**Real-time RT-PCR primers and probes**

A conserved region in sequence coding the N protein was identified and selected for the development of a group-specific real-time RT-PCR for the detection of Simbu serogroup viruses. A single set of primers was designed, based on the published sequencing data of twenty-eight out of thirty-two Simbu serogroup members, in order to amplify a product of 122bp. Two TaqMan® MGB probes labelled with different dyes were developed to allow for the distinction between phylogenetic clades. Both primer and probe sets are degenerate, as this genetically diverse group of viruses presented multiple nucleotide mismatches after alignment (Table 3 and Figure 2).

**Sequencing**

The S segment of the DVTD's Simbu serogroup isolates, AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV, which represent the prototype strains, Ja Gar 39, SA Ar 53, An 10107, IG 10310, An 5550, SA An 4165 and An 9398 respectively, as well as two other South African isolates of SHUV and SABOV, 8912 and Cu 1/70 respectively, were sequenced for the first time and the ORFs compared with published data. AKAV showed 5% nucleotide variation relative to Ja Gar 39 (GenBank® accession numbers AF034939 and AB000852). SIMV was 100% identical to SA Ar 53 (NC018477) and varied 0.3% to (HE795110). The two sequenced strains of SHUV, An 10107 and 8912, were identical to each other and varied by 0.99% when compared to the prototype strain (HE800143). SHAV was 100% identical to An 5550 (NC018464 and HE795107). INGV varied 0.14% compared to SA An 4165 (KF697141). SABOV strain An 9398 was 100% identical to a published sequence (HE795098) and varied by 0.14% to AF362396, and 1.7% to the other sequenced strain Cu 1/70. No reference to SATV strain IG 10310 sequence was found in GenBank®, but was 100% identical to two sequences (NC018462 and HE795104) published by Goller and colleagues (Goller *et al.*, 2012).

**The group-specific RT-qPCR assay**

**Development**

**Pilot run**

The group-specific RT-qPCR detected all the tested Simbu serogroup isolates. The quantification cycles ($C_q$) values for AKAV, SIMV, SHUV, SATV, SHAV, INGV and SABOV were 15,5, 15,0, 18,5, 17,0, 18,9, 25,7 and 24,7, respectively. No cross-reactivity was observed between the two different probes, which allow distinction

between the twos clades. Nevertheless, performance of both probes was quite distinct with probe Simbu_CladeAP showing much lower fluorescence intensity (Figure 3).

**Comparison with a pan-Simbu assay (Fischer *et al.*, 2013)**

In 2013, Fischer and colleagues published a real-time RT-PCR experiment for the broad-range detection of Simbu serogroup viruses, using fourteen out of the thirty-two members, which was named pan-Simbu. This intercalating dye based qPCR assay was at the time of publication the first available tool for the broad screening of Simbu viruses and was able to detect all the tested viruses, which included AINOV, AKAV, DOUV, PEAV, SABOV, SANV, SATV, SHAV, SHUV, SIMV, THIV, TINV and SBV from clade B and OROV from clade A. However, this system is not specific for Simbu serogroup viruses, as it was also able to detect Bunyamwera serogroup orthobunyaviruses. The pan-Simbu was reproduced at DVTD and again allowed the detection of all the tested viruses, AKAV, SIMV, SHUV, SATV, SHAV and SABOV from clade B and INGV from clade A. Nevertheless, using the same samples, the novel group-specific RT-qPCR showed much higher sensitivity (Table 4).

**Optimization**

Optimal concentration was defined as the lowest concentration of primers or probe able to generate a low $C_q$ with high efficiency. The lowest $C_q$ was obtained when using 800 nM primer concentration and no significant difference was observed between a 200 nM or 400 nM primer concentration. The detection of the target improved as the concentration of the probe increased. To limit the primer/probe concentrations in order to allow multiplex detection, a final primer concentration of 400 nM, a probe Simbu_CladeAP concentration of 500 nM and a probe Simbu_CladeBP concentration of 250 nM probe in the reaction was selected.

**Efficiency**

The amplification efficiency was calculated from the linear regression between the $C_q$ and $TCID_{50}$/reaction values obtained for each isolate: 99% for AKAV, 96% for SIMV, 96% for SHUV, 97% for SATV, 84% for SHAV, 93% for INGV and 110% for SABOV.

**Sensitivity**

The 95% limit of detection (LOD) of the assay, expressed by $TCID_{50}$/reaction, was determined by probit analysis for each virus was $10^{-3,61}$ for AKAV (95% confidence interval: $10^{-3,98}$ to $10^{-2,38}$), $10^{-2,38}$ for SIMV (95% confidence interval: $10^{-2,94}$ to $10^{-0,18}$), $10^{-3,42}$ for SHUV (95% confidence interval: $10^{-3,66}$ to $10^{-2,05}$), $10^{-3,32}$ for SATV (95% confidence interval: $10^{-3,56}$ to $10^{-1,95}$), $10^{-1,67}$ for SHAV (95% confidence interval: $10^{-2,07}$ to $10^{1,57}$), $10^{0,39}$ for INGV (95% confidence interval: $10^{0,03}$ to $10^{1,56}$) and $10^{-2,70}$ for SABOV (95% confidence interval: $10^{-3,01}$ to $10^{-1,59}$)

**Specificity**

The assay was shown to be specific for the detection of Simbu serogroup orthobunyaviruses, as no cross-reactions were observed *in vitro* with BVDV-1, BoHV-1, BTV-1, BEFV, RVFV, WSLV or PALV. *In silico* specificity analysis showed no cross-reactivity with other genetically related viruses which may be differential diagnosis along with Simbu serogroup viruses, namely those belonging to the Bunyamwera serogroup that also can cause abortion and teratology in ruminants.

**Repeatability**

The intra-run SD ranged from 0,50 and 0,71 for probe Simbu_CladeAP and 0,14 to 0,68 for probe Simbu_CladeBP. The inter-run SD ranged from 0,19 to 0,60 for probe Simbu_CladeAP and from 0,31 to 0,97 for probe Simbu_CladeBP. CV ranged from 1,36% and 2,01% for probe Simbu_CladeAP and 0,63% and 2,91% for probe Simbu_CladeBP detection.

**Discussion**

Like all negative-sense RNA viruses, the proofreading function of the *Orthobunyavirus* RdRp is absent, which generates significant genetic heterogeneity in virus populations (Elliott, 2014). In addition, this genetic diversity is suggested to be reasoned on the cosmopolitan distribution of the Simbu serogroup, as other *Orthobunyavirus* serogroups exhibit less genetic diversity, but a narrow geographical range (Saeed *et al.*, 2001a). The diversity between clades is expected, considering that 77% of clade A viruses occur in the New World and no clade B virus have been isolated in this region, as vector and host ranges influence the geographical spread of the microorganisms (Ladner *et al.*, 2014). The high degree of genetic diversity within the segments, particularly S and M, may be because the segments encodes for the N protein and surface glycoproteins (the major immunogens), respectively (Roman-Sosa *et al.*, 2016; Wernike *et al.*, 2017a). and therefore, it is possible that during the evolution of these viruses, the need to infect and alternate between mammal, bird or insect, may have played a role in the high diversity of the M segment (Saeed *et al.*, 2001a; Yanase *et al.*, 2012). Another explanation is that ancestral viruses may have undergone genetic reassortment (Bowen *et al.*, 2001; Gerrard *et al.*, 2004; Briese *et al.*, 2006; Aguilar *et al.*, 2011), resulting in progeny viruses with similar S segments, but diverse M segments (Saeed *et al.*, 2001a), as it is likely that the N protein and viral polymerase, which are essential for genome replication, to co-evolve together (Elliott, 2014). Only a few real-time RT-PCR assays have been developed for the molecular diagnosis of Simbu serogroup orthobunyaviruses, describing either virus-specific approaches in a simplex format (Bilk *et al.*, 2012; Van Eeden *et al.*, 2014b; Tauscher *et al.*, 2017), or broader approaches in duplex (Stram *et al.*, 2004b) or multiplex formats (Fischer *et al.*, 2013; Lee *et al.*, 2015; Shirafuji *et al.*, 2015; Naveca *et al.*, 2017). The majority of the assays target the S segment, which has the least amount of variation (Figure 1) (Saeed *et al.*, 2000; Nunes *et al.*, 2005; Acrani *et al.*, 2010; Vasconcelos *et al.*, 2011; Hang *et al.*, 2014; Cardoso *et al.*, 2015), but an assay targeting the L segment has also been described (Fischer *et al.*, 2013). The published methods describing the broader detection capacity of the Simbu serogroup viruses utilise either a SYBR® Green based chemistry able to recognise viruses from both clades, which is not absolutely specific (Fischer *et al.*, 2013), or a TaqMan® based chemistry that recognises only clade B viruses (Shirafuji *et al.*, 2015). In this paper, a group-specific TaqMan® based real-time RT-PCR is described, targeting the S segment and designed to distinguish viruses from both phylogenetic clades. The difference obtained in terms of fluorescence intensity may be explained with the amplification process itself, as multiple nucleotide mismatches were found between both primers and the clade A isolate stored at DVTD, Ingwavuma #2 BHK – 2/2/96, at both primers's 5' end. Efficiency values of less than 90% may be caused by *Taq* DNA polymerase contamination with inhibitors, inappropriate $T_M$ or poorly design primers, whereas efficiency values greater than 100% usually result from contamination with non-specifiic products or primer dimers (Tenreiro *et al.*, 2014).

**Conclusion**

Overall, this work shows the development of a novel group-specific one-step Taqman®-based RT-qPCR assay, with the ability to differentiate between two phylogenetic clades, which was optimized and laboratory validated to provide a rapid, sensitive and specific molecular diagnostic method for the broad detection of at least thirty-one viral pathogens, of which some have significant relevance in public and animal health. This assay showed more sensitivity, specificity and broad detection capacity when compared with other published methods for detection of Simbu serogroup viruses. Therefore, it can be a useful diagnostic tool, either in countries where a certain Simbu-associated endemic disease is present or in countries where the disease is exotic and demands a quick identification in case of suspected introduction. The assay can also be useful for the screening of populations that play an important role in the epidemiology of the Simbu serogroup viruses, such as arthropod vectors or wildlife animals, thus enabling the development of prevalence studies and consequently optimising the management and control of the diseases.

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## Tables

### 1. Simbu serogroup Orthobunyavirus isolates stored in the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Sciences, University of Pretoria.

| Name | Virus | Strain | $TCID_{50}$/ml |
|---|---|---|---|
| Akabane #2 Vero #3 BHK – 22/5/98[a] | AKAV | Ja Gar 39 | $1\times10^{4,9}$ |
| Ar 53 #2 BHK – 2/2/96[b] | SIMV | SA Ar 53 | $1\times10^{5,7}$ |
| Shuni #2 BHK #2 Vero – 22/5/98[c] | SHUV | An 10107 | $1\times10^{4,5}$ |
| Sathuperi #2 BHK – 2/2/96[d] | SATV | IG 10310 | $1\times10^{5,3}$ |
| Shamonda #1 Vero – 21/1/94[e] | SHAV | An 5550 | $1\times10^{5,3}$ |
| Ingwavuma #2 BHK – 2/2/96[f] | INGV | SA An 4165 | $1\times10^{6,5}$ |
| Sabo #2 Vero #1 BHK – 3/8/94[g] | SABOV | AN 9398 | $1\times10^{4,3}$ |
| 8912 #1 BHK – 2/2/96[h] | SHUV | 8912 | - |
| Cu 1/70 #1 Vero – 21/7/94[i] | SABOV | Cu 1/70 | - |

[a] **Isolated in Japan from pools of *Aedes vexans* and *Culex tritaeniorhynchus* mosquitoes in 1959 which was subjected to two passages in African green monkey kidney (Vero) cells and three passages in baby hamster kidney (BHK) cells.**

[b] **Isolated in South Africa from *Aedes circumluteolus* mosquitoes caught during 1955 and 1957 with 2 passages in BHK cells.**

[c] **Isolated in Nigeria from a bovine in 1966, with two passages in BHK cells and two passages in Vero cells.**

[d] **Isolated in India from pools of *Culex vishnui* mosquitoes in 1957, with two passages in BHK cells.**

[e] **Isolated in Nigeria from a bovine in 1965 with one passage in Vero cells.**

[f] **Isolated in South Africa from a spectacled weaver in 1959 with two passages in BHK cells.**

[g] **Isolated in Nigeria from a goat in 1966 with two passages in Vero cells and one passage in BHK cells.**

[h] **Isolated in South Africa from cattle during an outbreak of a formerly unrecognized disease in 1967 with one passage in BHK cells.**

[i] **Isolated in South Africa from *Culicoides* spp. in 1970 with one passage in Vero cells.**

### 2. Sequencing primers

| Name | Sequence (5'→3') | Location | Length | $T_m$ (ºC) | GC% |
|---|---|---|---|---|---|
| Akabane_AB289319F | GAACTCCACTATTAACTACGCATTG | 7-32 | 25 | 62 | 40 |
| Akabane_AB289319R | AAAGGTGTGCACCACATAGA | 785-805 | 20 | 62 | 45 |
| Simbu_ NC_018477F | AATGGCAAACCAATTCA | 24-41 | 17 | 55 | 35,3 |
| Simbu_ NC_018477R | GGCGTACAACACATAGA | 787-804 | 17 | 56 | 47.1 |
| Shuni_KU937313F | AGTGTAYTCCACTATAGAACAAAGC | 5-30 | 25 | 62 | 40 |
| Shuni_KU937313R | AGTGTGCTCCACATAGAACAAT | 828-850 | 22 | 62 | 40,9 |
| Sathuperi_HE795104F | CACTACTGAAATATGTCAAGCCAATTC | 32-59 | 27 | 63 | 37 |
| Sathuperi_HE795104R | CTCAACAGAAGCCTTGCAGTAT | 805-827 | 22 | 63 | 45,5 |
| Shamonda_NC018464F | CCACTATTAACTACAGAAATATGTCAAGCC | 11-41 | 30 | 64 | 36,7 |
| Shamonda_NC018464R | GGACCCGAAAGATGGTGAACTA | 848-870 | 22 | 64 | 50 |
| Ingwavuma_KF697141F | AGTAGTGTACTCCAC**W**ATTCAA | 1-23 | 22 | 59 | 36.4 |
| Ingwavuma_KF697141R | GTAGTGTGCTCCCAATTCA | 955-974 | 19 | 59 | 47.6 |
| Sabo_AF362396F | GTGTACTCCACTATTAACTACGTACC | 5-31 | 26 | 62 | 42.3 |
| Sabo_AF362396R | GAATTGGCGTGTCTCACATAGA | 791-813 | 22 | 62 | 45.4 |

### 3. Group-specific real-time RT-PCR primers and probes

| Name | Sequence (5'→ 3') | Location | Length | Tm (°C) | GC% |
|---|---|---|---|---|---|
| Simbu_F | TAGAGTCTTCTTCCTCAA**Y**CAGAAGA | 120-145 | 26 | 57 | 40 |
| Simbu_R | TA**Y**TGGGGAAAATGGTTATTAACCA | 218-242 | 25 | 58.6 | 34 |
| Simbu_CladeAP | VIC®-TACGT**M**AGACG**Y**CGAGG-MGB | 161-177 | 17 | 67 | 59 |
| Simbu_CladeBP | FAM™-T**Y**GGTTGTG**S**CGTCTT-MGB | 166-181 | 16 | 69 | 53 |

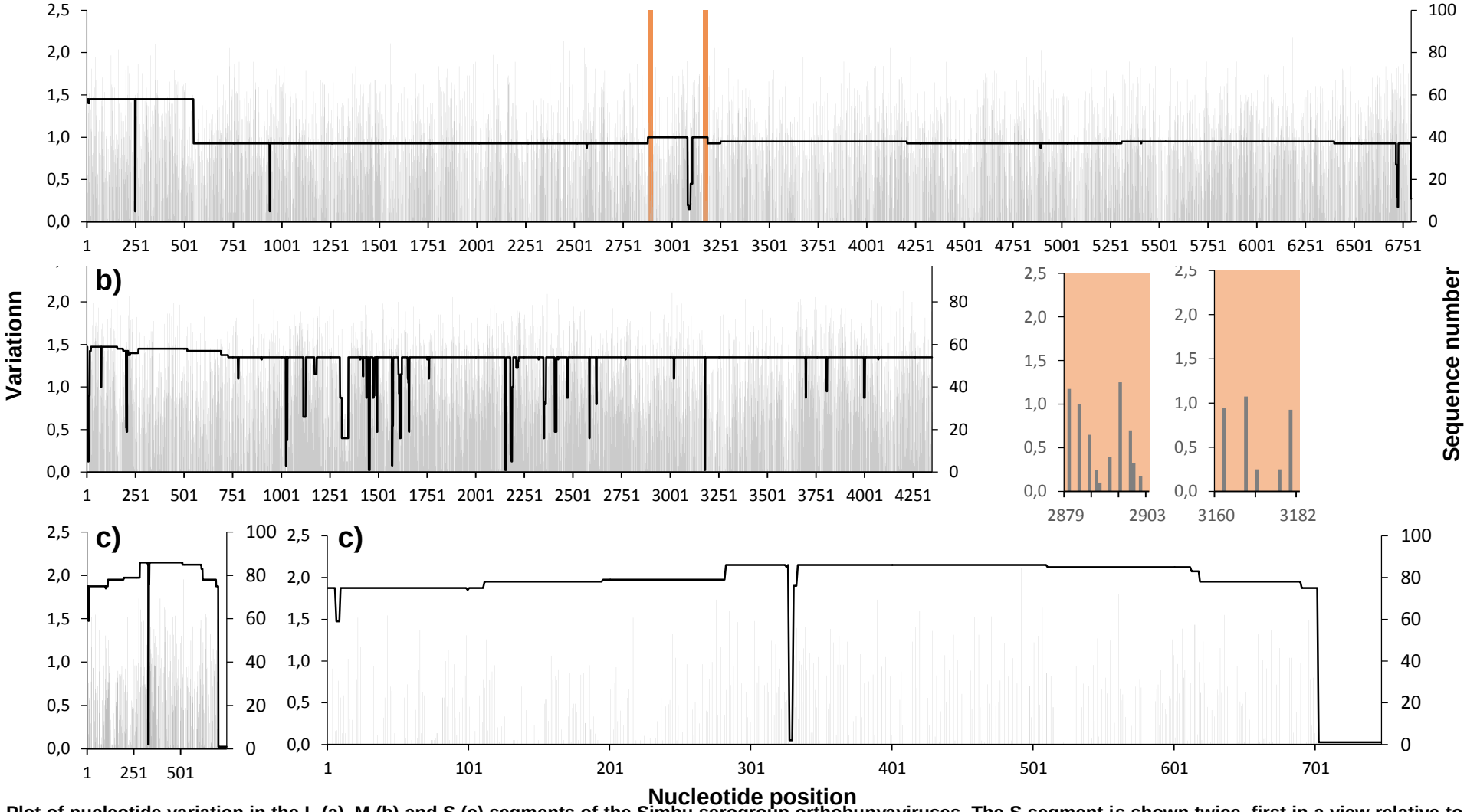
### 4. Comparison with a pan-Simbu assay

| Virus | Cq group-specific | Cq pan-Simbu | Cq Difference | Fold Difference |
|---|---|---|---|---|
| AKAV $10^{-1}$ | 17.61 | 32.13 | 14.52 | 23496 |
| SIMV $10^{-1}$ | 16.49 | 27.76 | 11.27 | 2465 |
| SHUV $10^{-1}$ | 19.14 | 33.37 | 14.23 | 19175 |
| SATV $10^{-1}$ | 27.91 | 33.75 | 5.84 | 57 |
| SHAV $10^{-1}$ | 21.52 | 28.75 | 7.23 | 150 |
| INGV $10^{-1}$ | 22.46 | 36.91 | 14.45 | 22431 |
| SABOV $10^{-1}$ | 26.79 | 32.21 | 5.41 | 43 |

**The table shows the Cq values obtained with the novel group-specific RT-qPCR and with the pan-Simbu using a 1:10 dilution of tissue culture viruses. * An efficiency of 100% has been assumed - theoretically, the group-specific RT-qPCR would be able to detect a 1:23496 dilution of AKAV $10^{-1}$ resulting in the same Cq value obtained with the pan-Simbu.**

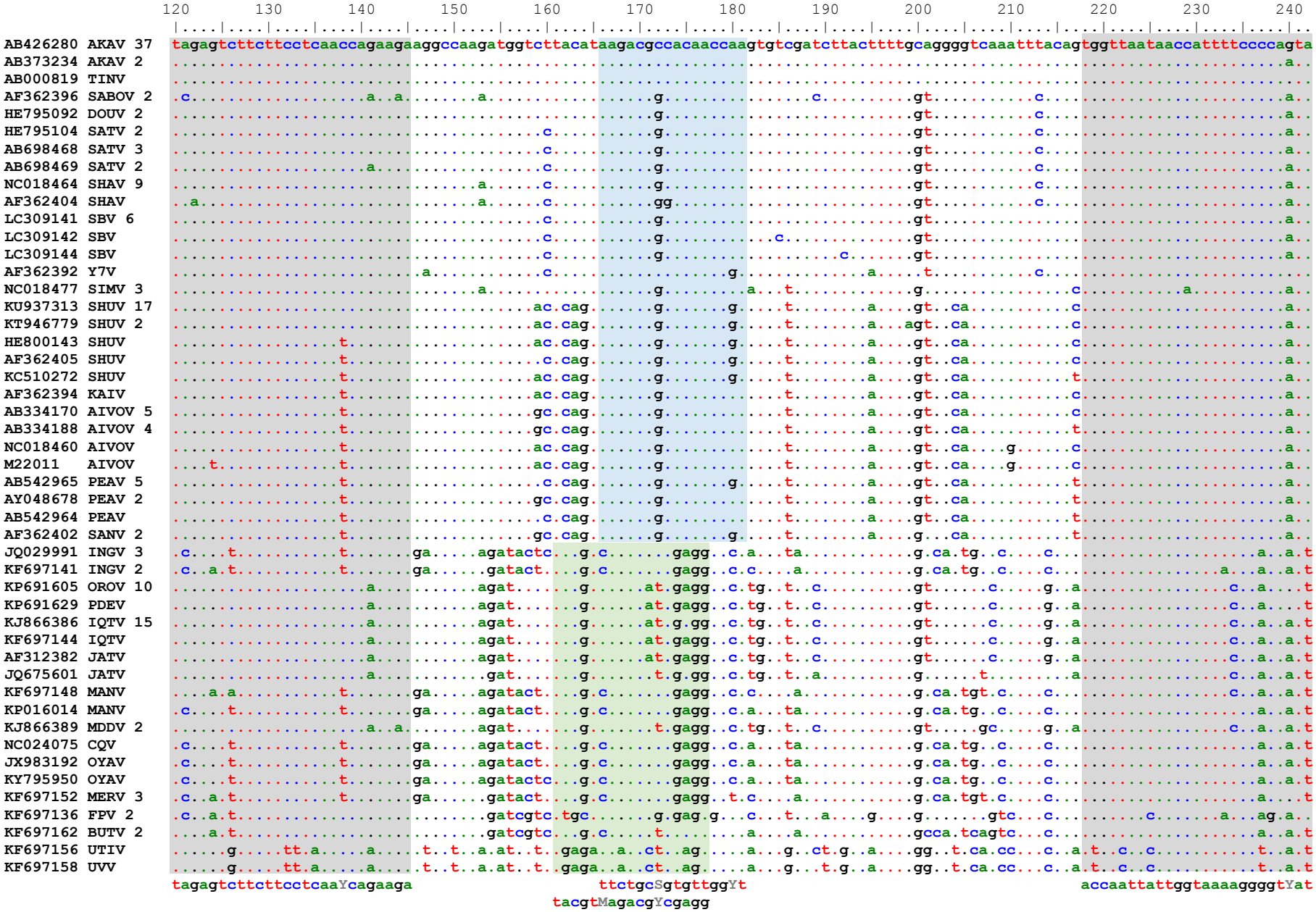
## Figures

### 1. Plot of nucleotide variation in the genome of the Simbu serogroup viruses



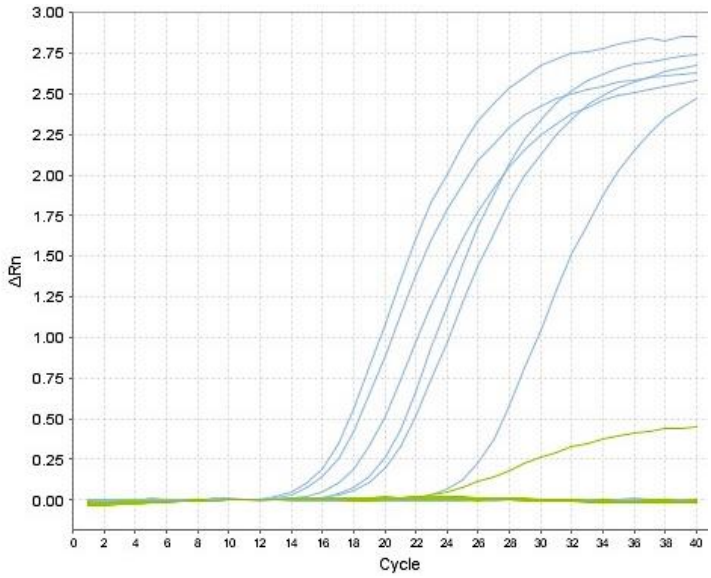

**Plot of nucleotide variation in the L (a), M (b) and S (c) segments of the Simbu serogroup orthobunyaviruses. The S segment is shown twice, first in a view relative to the other segments, and then in an expanded view to better illustrate conserved regions. Column height indicates the variation in each nucleotide position: the higher the column, the greater is the variation. All relevant sequences available in Genbank® were used, and the number of sequences analysed at each nucleotide position is indicated by the blu line. The orange columns indicate the primers used in the pan-Simbu real time RT-PCR assay (Fischer *et al.*, 2013)**

## 2. Simbu serogroup sequence alignment




**Genome region (120-242) targeted by the group-specific real-time RT-PCR, showing the nucleotide variation within the S segment. Numbers following the acronyms indicate the number of identical sequences. Dots indicate identity with the first sequence. Primers are represented by the grey shades, probe Simbu_CladeBP is represented by the blue shade (for Akabane, Tinaroo, Sabo, Douglas, Sathuperi, Shamonda, Schmallenberg, Yaba-7, Simbu, Shuni, Kaikalur, Aino, Peaton and Sango viruses detection) and probe Simbu_CladeAP is represented by the green shade (for Ingwavuma, Oropouche, Perdões, Iquitos, Jatobal, Manzanilla, Madre de Dios, Cat Que, Oya, Mermet, Facey's Paddock, Buttonwillow, Utinga and Utive viruses detection). Primer and probe sequences are indicated in the bottom.**

**3. Pilot run amplification plot**




**Amplification curves of the group-speciifc real-time RT-PCR plotted as fluorescence intensity (ΔRn) against cycle number showing successful amplification of AKAV, SIMV, SHUV, SATV, SHAV, SABOV (blue curves), and INGV (green curve). Negative control is represented by the flat line.**